In Memoriam

Ludwig Hektoen

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ARTIFICIAL RADIOACTIVE ANTIGENS

I. PREPARATION AND EVALUATION OF S^{35}-SULFANILIC ACID-AZO-BOVINE-γ-GLOBULIN

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Theories of the mechanism of antibody synthesis which have most general acceptance and which are most directly amenable to experimental investigation propose that the antigen, or some high molecular weight fragment, is present at the site of antibody synthesis and exerts a direct controlling influence.[1,2] This suggests that investigation of the fate of antigens in vivo would be a profitable approach to the problem of the mechanism of antibody synthesis.

Studies on the qualitative localization of a variety of antigens which can be identified in tissues indicate that antigenic material is quickly removed from the blood stream and concentrated chiefly in the liver and spleen.† (For reviews of this work see Coons and Kaplan,[3] and Taliaferro.[4]) The only extensive quantitative studies on antigen distribution and rate of elimination which have been published are those of Haurowitz and Breinl[5] and Libby and Madsen.[6] The former authors injected an arsanilic acid-azo-protein into rabbits and determined the arsenic content of the tissues at various times after injection. This study was restricted by the tedious arsenic assay, the limits of detecting arsenic and the storage of arsenic in certain tissues of particular interest with respect to antigen localization. The distribution and rate of elimination of tobacco mosaic virus labeled metabolically with P^{32} was studied by Libby and Madsen[6] in mice. They also compared the antibody titer in the serum with the concentration of P^{32} in the liver. Interpretation of the results was complicated by the rapid uptake of P^{32} from the disintegrating antigen by normal tissue constituents.

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* Public Health Service Research Fellow of the National Heart Institute, March through August 1950. This report is taken from a dissertation submitted to the Division of Biological Sciences of the University of Chicago in partial fulfilment of the requirements for the degree of Doctor of Philosophy, September, 1950.

† Since this paper was submitted for publication, observations on the fate of bovine globulin labeled with small amounts of I^{131} have been published which indicate that in the rabbit this antigen is removed from the blood much more slowly than has been observed for proteins containing azo-coupled haptens and that it shows little tendency to be concentrated in any of the tissues studied. (See Dixon, F. J., Bukantz, S. C., and Dammin, Q. T. 1951, Science **113**: 274–276.)

1. Mudd, S. 1932, J. Immunol. **23**: 423–427; Alexander, J. 1932, Protoplasma, **14**: 296–306; Breinl, F. and Haurowitz, F. 1930, Z. physiol. Chem., **192**: 145–157.
2. Pauling, L. 1940, J. Am. Chem. Soc. **62**: 2643–2657.
3. Coons, A. H. and Kaplan, M. H. 1950, J. Exper. Med. **91**: 1–13.
4. Taliaferro, W. H. 1949, Ann. Rev. Microbiol. **3**: 159–189.
5. Haurowitz, F. and Breinl, F. 1932, Ztschr. f. physiol. Chem. **205**: 259–270.
6. Libby, R. L. and Madison, C. R. 1947, J. Immunol. **55**: 15–26.

A prime requisite for long term, quantitative studies of the process of immunization in vivo is a suitable tagged antigen. With the present availability of radioactive isotopes, they are obviously the material of choice for tagging an antigen for this work which is essentially a highly specialized metabolic problem. Such an antigen should meet certain minimum requirements. The whole antigen should be soluble and a good antibody producer. The radioactive element should have a relatively long half-live and no γ radiation, should be stably bound to the antigenic moiety and should be in a strong determinant group which could not be lost without markedly altering the antigen. The trace element itself or the compound in which it is contained should be metabolically inert and rapidly excreted when cleaved from the intact antigen. Material labeled by metabolic incorporation of isotope will probably be unsuitable because of susceptibility to metabolic degradation. The utility of iodinated proteins is limited by the short half-life and γ radiation of I^{131}. Iodotyrosine would also probably give rise to labeled thyroxine and other compounds which would remain in the tissues.[7]

Sulfanilic acid-azo-bovine γ globulin (SAγG) labeled with S^{35} in the sulfanilic acid (SA) appeared likely to fulfill these requirements. Some studies with S^{35}-sulfanilic acid-azo-albumin in the rabbit have recently been reported at meetings of the Society of American Bacteriologists by Garvey et al.[8] The preparation of S^{35} AγG and an evaluation of its suitability for use in immunochemical and related investigations are reported in the present paper.

7. Gross, J. and Leblond, C. P. 1950, J. Biol. Chem. **184**: 489–500.
8. Garvey, J. S., Stahley, G. L. and Meyers, W. G. 1949, Abstracts of papers, The Society of American Bacteriologists, 49th meeting; Garvey, J. S., Dodd, M. C., Stahley, G. L. and Meyers, W. G. 1950, Abstracts of papers, The Society of American Bacteriologists, 50th meeting.

MATERIALS AND METHODS

Mice were from Carworth farms and weighed 25 to 30 g at the time of injection. After being injected with radioactive material, they were kept in three liter beakers equipped with a false bottom of stainless steel wire screen and ordinary heavy wire screen tops. They were fed either a pelleted laboratory chow from Ralston Purina Co., St. Louis, Missouri, or a similar pelleted complete dog food from Dixie Mills Co., East St. Louis, Illinois. To prevent excessive spillage into the bottom of the cage, food was put in wire mesh cylinders having 4 legs on the bottom and capped with a beaker having a piece of rod sealed to its bottom. The legs extending through the false bottom of the cage and the rod through the cover held the feeder in place. Mice were injected via the tail vein with a one ml precision type syringe using a glass apparatus which provided transillumination of the tail making the vein and the injection readily visible. This apparatus was suggested by one described by Nickson and Barkulis.[9] Two syringes which had been calibrated gravimetrically were used for all radioactive injections. Series of three or more one-tenth ml injections were probably always within 3% of the same volume. Mice were lightly etherized and bled from the axillary artery into a pocket formed by the skin. Tissues were taken in tared, cork stoppered tubes.

Preparation of samples for radioassay

Tissues.—Tissues were routinely counted directly as dried homogenates plated on copper disks. Except for livers, each organ from an entire group of autopsied mice was pooled, weighed to 0.01 g and placed in a glass homogenizer. Livers from each group were weighed, and a representative sample of approximately three g was treated as were the other organs. The tissue was assumed to have a density of one and sufficient distilled water was added to give a final homogenate containing approximately 0.3 g of fresh tissue per ml. Ten Broeck[10] homogenizers from the H. S. Martin Company, Evanston, Illinois and Scientific Glass Apparatus Company, Bloomfield, New Jersey were used because they lasted longer than the Potter-Elvehjem type. The pestle was turned at about 160 r.p.m. through friction

9. Nickson, J. J. and Barkulis, S. S. 1948, Science, **107**: 229–230.
10. Ten Broeck, C. 1931, Science, **74**: 98–99.

from a rubber stopper on a rod held in a drill press. The tendency of such homogenates to shrink into the center of the sample disk during drying was overcome by adding one mg of "Papain Merck NF" in a 20% aqueous suspension per ml of final homogenate and incubating at 37 C for 4 to 12 hours depending on the tissue. The specific activity of the tissues was corrected for papain solids. One ml aliquots were plated within a 10 cm^2 circle scribed on oxidized copper disks essentially as described by Hogness et al for biological fluids.[11] If the homogenates tended to overflow the circle, a trace of silicone stopcock lubricant was applied around the outer edge. After preliminary drying on a turntable under a hot air blower and infra red lamp, they were put in an oven at 70 to 80 C for at least 4 to 6 hours and were kept in a desiccator over calcium chloride until weighed and counted. Determinations on duplicate liver homogenates were usually within 3% of the mean. These methods of preparing and plating the tissue homogenates are based in part on unpublished procedures of F. E. Kelsey.

When the tissues were of very low specific activity, the homogenates were fused with sodium peroxide according to the method of Bailey,[12] except that the scale was increased to allow for a sample of about two g of fresh tissue. After S^{35} was recovered quantitatively as barium sulfate and weighed, samples were prepared as described below.

Barium sulfate.—The problem of preparing uniform samples from suspensions of insoluble material[13] was considerably simplified by the use of a suspension stabilizing agent. The purified salt free grade of high viscosity carboxy methoxy cellulose, CMC 70H, was supplied by Hercules Powder Company, Wilmington, Delaware. An aqueous solution of 5 to 6 mg/ml was homogenized in a Waring blendor, clarified by centrifugation, adjusted to 4.8 mg/ml, stored in the refrigerator and diluted to 1.2 mg/ml when needed. The diluted solution maintained its viscosity for several weeks if refrigerated. This material is useful in preparing samples of various substances from both aqueous and certain non-aqueous solvents.

Whenever S^{35} was determined as barium sulfate, the precipitate from hot acid solution was washed three times with distilled water by centrifugation, suspended in 0.2 ml of water, one ml of the above CMC solution added and one ml of the resulting mixture plated using a calibrated dropper. The specific activity of the barium sulfate was corrected for the CMC present in the final sample. Counts on replicate samples prepared with CMC were statistically less variable than counts on plain barium sulfate samples.

Blood.—To prevent blistering and flaking of blood when plated directly, one ml of an animal glue solution was added to 0.2 ml of oxalated blood, and one ml of the resulting mixture was plated. Such a sample gives about 45% of the maximum count which would be obtained if the blood could be plated directly as a thick sample. A satisfactory glue solution which is stable for at least 3 or 4 years was prepared by soaking one part high grade glue in one part water and liquefying by adding one part ethylene chlorohydrin.[14] For use this solution was diluted with water to 45 mg/ml.

Feces.—Radioactivity in the feces was practically all water soluble and was determined by plating extracts of air dried feces obtained by soaking them several days in the refrigerator in about 20 parts of water, stirring vigorously for 3 to 5 minutes and centrifuging a portion. When two or three mice were kept in the beaker cages to be described later, and samples were taken every 24 hours or less with layers of filter paper in the bottom to soak up urine, there was a maximum of one to two percent of the urine collected with the feces.

Urine.—The filter paper in which the urine was collected (together with spilled food) was soaked overnight in the refrigerator in 100 ml of water. A portion of the resulting solution was filtered through dry paper, and one ml aliquots were plated.

Radioassay procedures

Radioactivity was determined with an internal Geiger counter of the type described by Kelsey.[15] The counting setup was usually checked daily with a C^{14} standard giving about 6500 c.p.m. Samples which gave over 1000 c.p.m. were corrected for resolving loss (rarely more than 5% of the count). Empirical curves were prepared for self absorption corrections. Activities were calculated from averages of at least

11. Hogness, J. R., Roth, L. J., Leifer, E. and Langham, W. H. 1948, J. Am. Chem. Soc. **70**: 3840–3842.
12. Bailey, K. 1937, Biochem. J. **31**: 1396–1405.
13. Calvin, M., Heidelberger, C., Reid, J. C., Tolbert, B. M. and Yankwich, P. F., 1949, Isotopic Carbon, New York, John Wiley & Sons, Inc. pp. 104–122.
14. Borkenhagen, L. K., Armour and Company Research Laboratories, Chicago. Private communication.
15. Kelsey, F. E. 1949, Science, **109**: 566–567.

three counts which met Chauvenet's criterion for statistical reliability.

The μc used to express quantities of radioactivity is based on the reported value for the first shipment of $H_2S^{35}O_4$ obtained from Oak Ridge. The average conversion factor from counts in barium sulfate to μc was $2.82 \pm .02^* \times 10^7$ c.p.m./μc/mg barium sulfate in 10 cm^2 thick sample on copper disks as described above. For the computation of activity in samples, a single factor to convert to μc, correct for decay and allow for day to day fluctuations in the counting system, was obtained by counting a $BaS^{35}O_4$ standard every day. This factor was altered to allow for the absorbing properties of the different sample materials, so that all results are expressed in terms of the Oak Ridge μc. Recovery of a known amount of activity mixed with the different materials sampled was always within 10% and usually within 5% of the mean. This recovery was satisfactory for the present work, since the most important comparisons were between samples all counted directly as homogenates.

S^{35}-labeled sulfanilic acid ($S^{35}A$)

Pure aniline acid sulfate was allowed to exchange with $H_2S^{35}O_4$ and then converted into SA essentially according to the instructions of Huber.[16] In 50 mg batches about 95% of the S^{35} used was incorporated into SA. When one-half g portions of orthanilic, metanilic and sulfanilic acids were dissolved in separate aliquots of a solution of the product and recrystallized 4 times, the specific activity of the sulfanilic acid was at least 500 times that of the ortho or meta isomers. After two recrystallizations from carrier sulfate, less than 0.2% of the S^{35} of the product was in the form of free sulfate. Carrier free $H_2S^{35}O_4$ solution was obtained from the Isotopes Division, U. S. Atomic Energy Commission, Oak Ridge, Tennessee.

EXPERIMENTAL RESULTS

Exchange of S^{35} between sulfanilic acid and sulfate

In order to establish the reliability of in vitro manipulations and analytical procedures, the possibility of an exchange of S^{35} between SA and $SO_4^=$ in 0.1 *N* hydrochloric acid neutral or 0.1 *N* sodium hydroxide solutions at 37 and 80 C was tested between 12 and 90 days. One series of three flasks was set up having S^{35} in the SA and a similar series with S^{35} in the $SO_4^=$. Samples were taken after 12 and 35 days at 37 C and after an additional 55 days at 75 to 85 C.

For the $S^{35}A \rightarrow SO_4^=$ series a control sample of barium sulfate showed a maximum of 0.15% of the total S^{35} to be in the sulfate on the first day, and samples at 12, 35 and 90 days averaged 0.18, 0.18 and 0.32% respectively with no significant pH effect. For the $S^{35}O_4^= \rightarrow SA$ series a control sample of SA showed an apparent exchange of 0.12% of the SA sulfur with $S^{35}O_4^=$ if all free $S^{35}O_4^=$ is assumed to have been eliminated by recrystallization of the SA. The average apparent exchange at 12, 35 and 90 days was 0.05, 0.05 and 0.65% and no pH effect was observed.

These data indicate that any possible cleavage of SA to yield sulfate or any spontaneous exchange of sulfur as $SO_4^=$ with SA sulfur does not take place at a rate sufficient to be of concern in the contemplated applications.

S^{35}-sulfanilic acid-azo-bovine-γ-globulin

Preparation—Radioactive antigens were prepared with a view to holding bacterial contamination to a minimum, but aseptic technique was not used, and the antigens were not sterilized prior to injection. One of the labeled antigens, however, was found to be sterile by culture on blood agar, in thioglycollate veal infusion broth and in plain veal infusion broth.

Fifty-two mg of radioactive SA in 8 ml of cold water plus 0.5 ml 3*N* hydrochloric acid was diazotized with 2.1 ml of 1% sodium nitrite for half an hour, after which 0.15 g of urea was added. In another half hour the solution was neutralized with 0.3 g sodium acetate

* Standard errors will always be given unless stated otherwise.

16. Huber, W. 1932, Helv. Chim. Act. **15**: 1372–1383.

and slowly added to 400 mg of bovine-γ-globulin (bovine plasma fraction II from Armour laboratories, Chicago, Illinois) in 10 ml of saline. The solution was adjusted to pH 8 ("Alkacid" pH paper) with 0.5*M* sodium carbonate and refrigerated overnight. The azo protein was precipitated at pH 4 with molar acetic acid, was kept in the refrigerator 8 to 10 hours and centrifuged in the cold. The precipitated antigen was suspended and recentrifuged from very dilute acetic acid and then suspended in saline and dissolved at pH 6 to 7 with 0.5*M* sodium carbonate. After the precipitation, washing and solution was repeated, the product in 17 ml saline at pH 7 to 7.4 was dialyzed in the cold in Visking cellulose casing for approximately 12-hour periods with stirring against 7 changes of 1 L each of saline containing 0.01*M* phosphate buffer, pH 7.4. One-half g of SA adjusted to pH 7 and one g sodium sulfate were added to the second and third dialysates.

Nonradioactive antigen for immunization and testing was prepared similarly in 5 g lots and was sterilized by passing through a Seitz filter.

Yields calculated from determination of solids less ash were 78 and 81% of total globulin plus SA for two large-scale nonradioactive preparations. The organic solids from these preparations were 15.0 and 15.4% Kjeldahl nitrogen respectively (determined by Groman's modification[17]). For two small scale radioactive preparations the yields calculated from Kjeldahl nitrogen were 83 and 84%.

The percent SA in two radioactive preparations after dialysis was 3.82 and 6.12 calculated from determinations of radioactivity. Assuming a molecular weight of 160,000 for the globulin, these figures would give 37 and 60 molecules of SA per molecule of γ-globulin.

Stability.—The rate of in vitro breakdown of three antigen preparations at 0 C and 37 C was measured in terms of the appearance of dialyzable S^{35} in 4 experiments. The antigen solutions in Visking casings (with an air space) were rotated end over end in successive portions of 50 to 100 times their volume of plain saline or saline buffered with 0.01*M* phosphate, pH 7.4. The relative rate of appearance of S^{35} in the dialysate decreased throughout the period of observation (30 days in one experiment), but after the first few days the rate of decrease became small.

One preparation which had been stored at 0 to 3 C for 42 days after an initial dialysis lost 10.1% during the first day of dialysis at 0 C (0.25% per day calculated for a first order decay for 42 days) but only 0.2% per day for the next 3½ days. When the temperature was then raised to 37 C, 8% of the remaining S^{35} dialysed in 10 hours, 4.5% in the next 5 days and only about 0.6% per day from 5 to 30 days. Thus, the equilibrium rate of breakdown for an aged preparation at pH 7.4 appears to be about 0.25% per day at 0 C and 0.6% per day at 37 C. If these are assumed to be true decay constants, they indicate a half-life of 300 days at 0 C and 110 days at 37 C.

Antigenicity.—Sulfanilic acid is a strong antigenic determinant when incorporated into an azoprotein and causes the formation of highly specific antibodies in rabbits, as shown by the work of Landsteiner.[18]

Immune serums were tested by the interfacial precipitin method. Serums from rabbits given 12 intravenous injections of SAγG had titers over 1:400,000

17. Groman, N. B. 1951, J. Infect. Dis. **88**: 126–150.

18. Landsteiner, K. 1947, The specificity of serological reactions, Revised ed., Cambridge, Harvard Univ. Press, pp. 310.

against SAγG and up to 1:40,000 against SA-azo-ovalbumin. Of greater importance to the work reported in paper II[19] is the fact that most mouse serums also showed a precipitin titer against SAγG, although with no demonstrated specificity for the SA component. From 70 mice immunized as described in paper II 48% of the serums were probably over 1:100,000 in titer (includes 12 mice dead of shock) and 80% were over 1:200. When a lapse of 4½ days intervened between intraperitoneal and intravenous injections, and no desensitizing injection was given, 10 of 25 mice went into severe anaphylactic shock and 7 died. This mortality closely approximates the observations of Weiser et al[20] when 5 to 15 days elapsed between intraperitoneal and intravenous injections of egg albumin.

Excretion of possible S^{35}-containing breakdown products of $S^{35}A\gamma G$

Free $SO_4^=$ or SA although unimportant with respect to the immune response, might conceivably be formed in vivo from SAγG. It is thus necessary to know whether radioactive $SO_4^=$ or SA might be stored in the tissues in sufficient concentration to interfere with tracing the radioactivity of S^{35} AγG.

The excretion of $S^{35}O_4^=$ during 5 days following oral and intraperitoneal administration has been studied in rats.[21,22] The concentration of S^{35} in the tissues was low, and 95% was recovered in the excreta in 5 days. However, since some applications of this antigen will require tracing the long term retention of small amounts of S^{35} introduced intravenously into mice, the rate of excretion of S^{35} injected both as $SO_4^=$ and as SA was investigated.

For this experiment mice were injected via the tail vein as follows: 0.1 ml saline containing .01 mg sodium sulfate (about 10% of the total blood sulfate as reported for man) with about 1.8 μc S^{35} into 12 mice; 0.2 ml saline containing 0.2 mg SA (adjusted to pH 7) with about 4.5 μc S^{35} into 6 mice; and for comparison 0.1 ml saline containing 0.76 mg SAγG with about 0.24 μc S^{35} into 34 mice. This last series is from part of the second experiment described in detail in paper II.[19] Groups of 2 to 5 mice from a given series were then placed in appropriate cages, and urine and feces samples and various tissues were collected.

The "relative specific activity" of the tissues in terms of the percent of the dose per mouse per g of dry tissue is presented in figure 1. The activity in the liver, spleen and kidney from free sulfate after one day is only 1.1, 6.5, and 18% respectively of that from SAγG, and the rate of disappearance of the activity from sulfate is more rapid than that from SAγG throughout the 40 days studied. The concentration in the bone marrow and blood is about the same as from SAγG, but that in the brain is considerably higher (compare table 1 of paper II.[19] These data for sulfate are essentially similar to those reported in the studies cited above (Dziewiatkowski[21] and especially Singher and Marinelli[22]).

The excretion of SA was so rapid that by the end of one day the relative concentrations in the liver, spleen and kidney were only 0.005 to 0.05% of those from SAγG.

Both $S^{35}O_4^=$ and S^{35}A were recovered in the excreta to the extent of about 90% by the end of the first day and only

19. Ingraham, J. S. 1951, J. Infect. Dis. **89**: 117–129.
20. Weiser, R. S., Golub, O. J. and Hamre, D. M. 1941, J. Infect. Diseases, **68**: 97–112.
21. Dziewiatkowski, D. D. 1949, J. Biol. Chem. **178**: 197–202.
22. Singher, H. O. and Marinelli, L. 1945, Science, **101**: 414–415.

1 to 2% more by the 4th day. Direct analysis of the carcasses showed that less than 1% of the S^{35} was retained, so that the total analytical recovery of the original dose was about 92%.

Analytically significant amounts of S^{35} from sulfate were retained in the periods after injection of S^{35} AγG is probably not due simply to $S^{35}O_4^{=}$ or S^{35}A derived from the antigen.

DISCUSSION

The work reported in this paper shows that SAγG is an easily prepared, stable

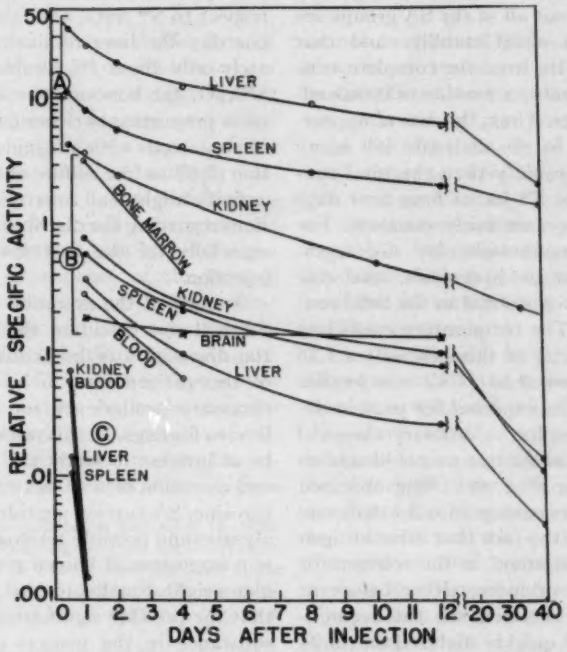

FIG. 1.—Disappearance of (A) SAγG, (B) $SO_4^{=}$ and (C) SA from tissues of normal mice. "Relative specific activity" is the percent of the total injection per mouse per g dry weight of tissue.

liver, spleen and kidney for as long as 40 days. However, it is probable that little or none of the S^{35} AγG breaks down in vivo to yield $S^{35}O_4^{=}$. In 6 urine samples from groups of mice injected with S^{35} AγG, an average of only 1.3% of the total S^{35} was precipitated by barium chloride in the presence of carrier sulfate either before or after hydrolysis with hydrochloric acid. Therefore, it may be concluded from this experiment that S^{35} retained in the tissues for extended antigenic material satisfactory for long term quantitative studies of the process of immunization in vivo. The suitability of this substance is further illustrated by the work reported in paper II[19].

The 60 molecules of stably bound SA per molecule of γG in one of the radioactive antigens is equal to almost 1 per tyrosine residue provided the data of Brand et al[23] for human-γ-globulin

23. Brand, E., Kassel, B. and Saidel, L. V. 1944, J. Clin. Investigation, 23: 437–444.

holds for bovine-γ-globulin. According to the work of Boyd and Hooker,[24] these groups are only about 35% of the maximum number of diazo groups which might be introduced into such a protein molecule with its 60 tyrosine and 26 histidine residues. The results from the dialysis of the radioactive antigen indicate that not all of the SA groups are bound with equal stability and that loss of activity from the complete antigen may involve a number of independent processes. Thus, the rate of appearance of S^{35} in the dialysate fell many times more rapidly than the total concentration of S^{35} for as long as 9 days and then became fairly constant. For a simple mono-molecular disintegration process or hydrolysis, the rate should be proportional to the total concentration. The temperature coefficient for the velocity of this process (ca 1.26 per 10 C from 0 to 37 C) was smaller than would be expected for protein denaturation or for an ordinary chemical reaction. That the true rate of liberation of dialyzable S^{35} is not being obscured by delay in its passage into the dialysate is shown by the fact that after antigen solutions had stood in the refrigerator for 40 days or more, either before or after a previous dialysis, the accumulated free S^{35} quickly dialyzed out in 24 hours or less, and the rate of dialysis of S^{35} was then fairly constant.

Studies on the stability of SA toward decomposition to yield sulfate or to exchange S^{35} with free sulfate are significant only with respect to the reliability of in vitro chemical manipulations and analytical procedures. However, the rate of excretion of S^{35} from SA demonstrates that SA is practically inert metabolically when injected in the free state.

24. Boyd, W. C. and Hooker, S. B. 1934, J. Biol. Chem. **104**: 329–337.

Although SA was eliminated from the tissues so rapidly that it would not interfere in any long term study, such is not the case for sulfate. For example the rank of the liver, spleen, kidney and bone marrow with respect to the relative degree of retention of S^{35} from $S^{35}O_4^=$ is in the opposite order to their rank with respect to S^{35} AγG, and, although after one day the liver retained proportionately only about 1% as much $S^{35}O_4^=$ as S^{35}AγG, the bone marrow retained the same proportion of either one. Thus, use of an antigen with a significant proportion of S^{35} as free sulfate of high specific activity might lead to erroneous conclusions regarding the distribution of SAγG especially for the first few days after injection.

A study of the excretion of $SO_4^=$ and SA will not elucidate the problem of the disposition of breakdown products of the antigen itself in vivo but is a necessary preliminary for interpreting in vivo findings. In this respect, it would be of interest to study the distribution and excretion of such substances as SA-tyrosine, SA-tyrosyl peptides, SAγG dialysates and possibly SA-containing protein fragments of known average molecular weight. Finally, it must be admitted that the possible significance of a given substance in the process of antibody synthesis is as yet highly speculative, since little is known concerning the characteristics of antigenic material which are directly responsible for the stimulation of antibody formation.

SUMMARY

Sulfanilic acid-azo-bovine-γ-globulin labeled with S^{35} in the SA has been prepared and has been found suitable as an antigen for quantitative in vivo studies of the process of antibody synthesis.

ARTIFICIAL RADIOACTIVE ANTIGENS

II. THE METABOLISM OF S^{35}-SULFANILIC ACID-AZO-BOVINE-γ-GLOBULIN IN NORMAL AND IMMUNE MICE

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Salley and Libby[1] used the data of Libby and Madison[2] on P^{32}-labeled tobacco mosaic virus as an antigen in mice to develop kinetic expressions for the rate of decomposition of antigen in terms of the concentration of antibody. Although Salley and Libby obtained an equation which gave a satisfactory fit to the experimental data by assuming a first order dependence of the rate of antibody production on the concentration of antigen and first order rates of decomposition of both antigen and antibody, they made no claim to have proven such a mechanism. As is also true for equations describing the course of antigen-antibody reactions in vitro, the validity of the mathematical conclusions cannot be assessed without more complete experimental information. Other pertinent considerations and references are reviewed in my previous paper.[3]

In the first paper of this series, methods are given for preparing the antigen used in this study. In addition, the results of investigation of its stability, metabolic inertness of its radioactive label and other properties necessary to establish its suitability for the present studies are reported.

In the present work, observations have been made and quantitative data have been obtained with regard to the disposition of the antigen, sulfanilic acid-azo-bovine-γ-globulin (SAγG) labeled with S^{35} in the sulfanilic acid (SA), in normal and immune mice. Of particular interest are the quantitative differences in the metabolism of this antigen by normal and immune mice. The problem of demonstrating and measuring differences in the disposition of antigens in normal and immune animals has been of interest for many years. Haurowitz and Breinl[4] attempted, without success, to demonstrate such a difference in their work with an arsenic-labeled antigen. The paucity of published experimental work and the desirability of applying radioactive tracers to this problem are mentioned in a recent review.[5] In addition, it was found that

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1. Salley, D. J. and Libby, R. L. 1947, J. Immunol. **55**: 27–35.
2. Libby, R. L. and Madison, C. R. 1947, J. Immunol. **55**: 15–26.
3. Ingraham, J. S. 1951, J. Infect. Dis. **89**: 109–116.
4. Haurowitz, F. and Breinl, F. 1932, Ztschr. f. physiol. Chem. **205**: 259–270.
5. Grabar, P. 1950, Ann. Rev. Biochem. **19**: 453–486.

SAγG is probably not eliminated according to a single, continuous, first order process as was suggested for tobacco mosaic virus.[1] Finally, the distribution of SAγG within tissues and cells has been studied, and radioactive compounds remaining in the tissues for extended periods of time have been investigated.

METHODS

For precipitin titers 0.2 to 0.3 ml of blood was taken from an incision in one of the tail veins and collected directly in a 4x50 mm precipitin tube. The blood was allowed to clot overnight in the refrigerator in corked tubes and serum removed the next day or shortly thereafter. Interfacial precipitin tests were run using 0.02 to 0.03 ml of serum with antigen dilutions of 1:200; 1:1000; 1:10,000; and 1:100,000; and a saline control. This amount of serum was sufficient when the small tube recommended by Boyd[6] was modified by drawing out a tail on the bottom which could be stuck in the clay used to hold it. The final reading was made after two hours, but the tubes were observed frequently to aid in distinguishing weak reactions from a slight clouding which sometimes occurred at the interface. Optimal lighting and nonhemolized serum were helpful. Hemolysis of the blood was minimized by coating the tubes in which it was collected with "Drifilm," according to the method of Moore and Stein.[7]

Methods for handling the mice, autopsying them and collecting tissues and fecal and urine samples are outlined in paper I,[5] except for the following. Bone marrow was obtained by cleaning the femurs, cutting off the ends and forcing the contents out with a stainless steel wire into a micro-glass homogenizer. Intestines were cut loose from the mesentery, the contents expelled, the lumen rinsed with mammalian Ringer's solution and the excess fluid expelled before placing them in tared vials. Skeletal muscle was dissected from the thigh. Axillary and inguinal lymph nodes were analyzed.

Methods for localizing and identifying S^{35} compounds in the tissues will be described in the experimental section. Methods for carrying out radioassays are described in paper I.[5]

6. Boyd, W. C. 1947, Fundamentals of immunology, ed. 2, New York, Interscience Publishers, pp. 421.
7. Moore, S. and Stein, W. H. 1949, J. Biol. Chem. **178**: 53–77.

EXPERIMENTAL RESULTS

Disposition of S^{35} AγG in normal and immune mice

S^{35}AγG was injected intravenously into a series of normal and of immune mice, and its rate of excretion in the feces and urine was determined, as well as its concentration in the liver, spleen, kidney, lymph nodes, small intestine, mesentery, ovary, lung, adrenal, heart, brain, blood and skeletal muscle at various times after injection.

Two independent experiments yielded essentially similar results. Therefore only the more extensive and technically better standardized second experiment will be described in detail. For this experiment 25 to 30 g mice from Carworth Farms were used. A total of 111 mice, 54 immunized and 57 normal controls, were autopsied.

Mice were immunized by a series of 5 semi-weekly intraperitoneal injections of 1 mg nonradioactive SAγG in 0.1 ml of saline followed by two semi-weekly intravenous injections of 0.5 mg. Each intravenous injection was preceded the day before by an intraperitoneal desensitizing injection. Fatal anaphylactic shock occurred in 7 of 25 mice which were not given a desensitizing injection before the first intravenous injection. This course of injections was used to take advantage of the ease and greater effectiveness of intraperitoneal inoculation and still attain the distribution of antigen given by intravenous injection before administering the radioactive antigen. Each mouse was bled for a precipitin titration 3 to 4 days after the first intravenous injection. Serums from 58 mice were titered; 38% were over 1:100,000 and 76% were 1:200 or above. These mice were divided into 12 groups of 3 to 5 having precipitin titers representative of those in the whole population. Radioactive SAγG was in-

jected into both normal and immunized mice 4 days after the last immunizing injection. Each mouse received 0.76 mg with 0.238 μc S^{35} (ca 3.6×10^5 cpm in zero thickness in the assay system used). All immune groups and 4 normal* groups from which excreta was collected were kept in the beaker cages described in paper I.[3] The rest of the normal mice were kept in large cages and were selected at random, usually in groups of three, for autopsy.

For the first experiment 80 mice were jected with saline according to the schedule used for immunization in the second experiment, bled from the tail, injected with 1 mg of $S^{35}A\gamma G$ 4 days later and autopsied the next day. The average specific activity of their livers was 0.98 ± 0.03† compared to 0.93 ± 0.04 for a similar group of 11 mice given only the injection of $S^{35}A\gamma G$. On another occasion similar results were obtained for two groups of 5 mice each. Therefore it is very improbable that the operation of injection and bleeding could have

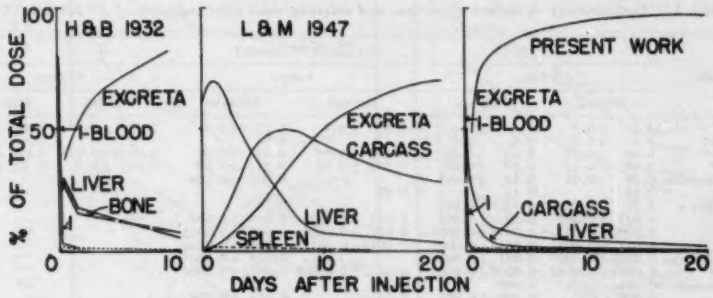

FIG. 1.—Distribution and elimination of labeled antigens. The first two graphs have been drawn from data given in the papers of Haurowitz and Breinl[4] and Libby and Madison.[2] The spleen is the broken line in each graph.

divided equally into 8 immune and 8 normal groups. Sixty-six were autopsied, the rest having died apparently from enteric infection. Most of the immunizations were done by intravenous injection before the intraperitoneal route was found to be more effective. Therefore, the immune mice had a lower average titer than in the second experiment. In this experiment the liver, spleen, kidney, lymph nodes and carcass only were sampled. Otherwise the first experiment was very similar to the second one.

As a control for the effect of injection and bleeding on the disposition of $S^{35}A\gamma G$ a group of 12 mice were in- been responsible for the differences between the livers of normal and immunized mice found in these experiments and reported in the section on the livers.

A general picture of the distribution and excretion of the identifying label in the present experiment and in the only previously reported work which is directly pertinent is given in the three graphs of figure 1. It is evident that there is no accumulation of activity from $S^{35}A\gamma G$ in the carcass with delayed excretion such as was reported by Libby and Madison[2] for P^{32}-labeled tobacco mosaic virus. Neither was

* These nonimmunized mice which have had only the single injection of $S^{35}A\gamma G$ will for convenience be referred to as "normal mice."

† Standard errors will always be given unless stated otherwise.

there any appreciable quantity stored in the bone as found by Haurowitz and Breinl[4] using an arsanilic acid azoprotein.

Recovery of S^{35} in urine and feces.— Excretion of S^{35} into the urine and feces of normal mice was slower than for immune, but approximately 75% of the injected S^{35} was eliminated within one day and 90% within 6 days by both normal and immune groups. The urine contained about 85% of the excreted immune mice at 1, 4 and 20 days are given in table 1 arranged in order of decreasing percent of the S^{35} in the normal series at one day. Quantitatively the liver is by far the most important of any organ examined; at one day it had more of the activity than all the rest of the body and over 30 times as much as the kidney or spleen, the next two most active organs.

Figure 2 presents a summary of the relative concentrations and rates of

TABLE 1.—*Radioactivity in tissues of normal and immune mice after injection of S^{35} labeled SAγG.*

Tissue	% of total S^{35} injected											
	1 day				4 days				20 days			
	Normal		Immune		Normal		Immune		Normal		Immune	
Liver*	14.1	±0.7	11.9	±0.6	8.0‖	±0.9	6.8‖	±0.2	2.4‖	±0.3	1.9‖	±0.3
Carcass†	8.6	±0.3	9.0	±0.3	3.1‖		2.9‖				0.67‖	±0.02
Kidney*	0.46	±0.02	0.47	±0.08	0.21‖	±0.07	0.15‖	±0.01	0.062‖	±0.015	0.05‖	±0.01
Spleen*	0.44	±0.12	0.62	±0.06	0.24‖	±0.03	0.27‖	±0.05	0.12‖	±0.04	0.10‖	±0.005
Small Intestine	0.38	±0.01	0.13	±0.009			0.064	±0.008				
Blood‡	0.16		0.12		0.05							
Mesentery	0.16	±0.01	0.13	±0.01								
Lung	0.037	±0.002	0.10	±0.003	0.016	±0.002	0.016	±0.002				
Ovary	0.030	±0.001	0.0074	±0.0006	0.017	±0.0005	0.0054	±0.0005				
Heart	0.013	±0.0007	0.0068	±0.0007	0.007‖	±0.0015	0.0064	±0.0002				
Lymph§ Nodes	0.0093	±0.0005	0.021	±0.0006			0.023	±0.0007				
Adrenal	0.0046	±0.0003	0.0035	±0.0002	0.0027	±0.00008	0.0015	±0.0002				
Bone Marrow	0.004		0.006									
Brain	0.003		0.009	±0.003	0.003		0.008	±0.004				
Skeletal Muscle												

Indicated errors of values:
As reported under "Radioassay Procedures" in paper I,[3] the mean value for the deviation of duplicates from their average for the tissue analyses is less than 3%. This is the error indicated in the table except in the following cases: (a) The standard error of counting is reported where this error, calculated for Poisson series, exceeds 3%. (b) For those values which are averages of the 1st and 2nd experiments, the error indicated is the deviation of the individual values from the average.
* Value is average of 1st and 2nd experiments. Other tissues are from 2nd experiment only.
† Whole mouse less liver, kidney, spleen, lymph nodes and most of the blood. Analyzed in the 1st experiment only.
‡ Standard error is at least 10%. For computation, 25 to 30 g mice were assumed to have 2.5 ml of blood with 20% total solids.
§ Axillary and inguinal.
‖ At least 1 value (in most cases that for the 1st experiment) for these averages was obtained from a curve fitted to the experimental points by inspection.

activity from $S^{35}A\gamma G$ compared to over 99% from $S^{35}A$ and about 98% from $S^{35}O_4^{=}$. The average total direct analytical recovery of administered S^{35} in carcass, tissues and excreta was $95.0 \pm 3.4\%$ for 14 groups from the first experiment. The carcasses were not analyzed in the second experiment, but if they are assumed to be the same as for the first, the average recovery from 8 groups was $98.4 \pm 1.3\%$.

Concentration of S^{35} in the tissues.— Data for percent of the administered S^{35} in all tissues analyzed from normal and elimination of S^{35} for the first 5 days from organs of groups of 3 to 5 mice. Antigen is removed from the blood so rapidly that after 12 hours the specific activity of the organs will not be measurably influenced by the presence of small amounts of blood. The relative distribution of S^{35} is rather similar in normal and immune mice with generally somewhat higher concentrations in the normal except for the lymph nodes and possibly the lungs in which this trend is reversed.

The liver, spleen, lymph nodes and

kidney were studied in more detail than the other organs. The data for the relative specific activity in these organs for both normal and immune mice are graphed in figures 3 and 4. The specific activity of the normal tissue as compared to the immune is higher for the liver and probably also for the spleen; it is lower for the lymph nodes and shows no consistent difference for the kidney. The rate of elimination of S^{35} from all of these tissues decreased for the first 12 to 15 days, and then in the liver and spleen it became fairly constant. Further data might show a similar situation in the lymph nodes.

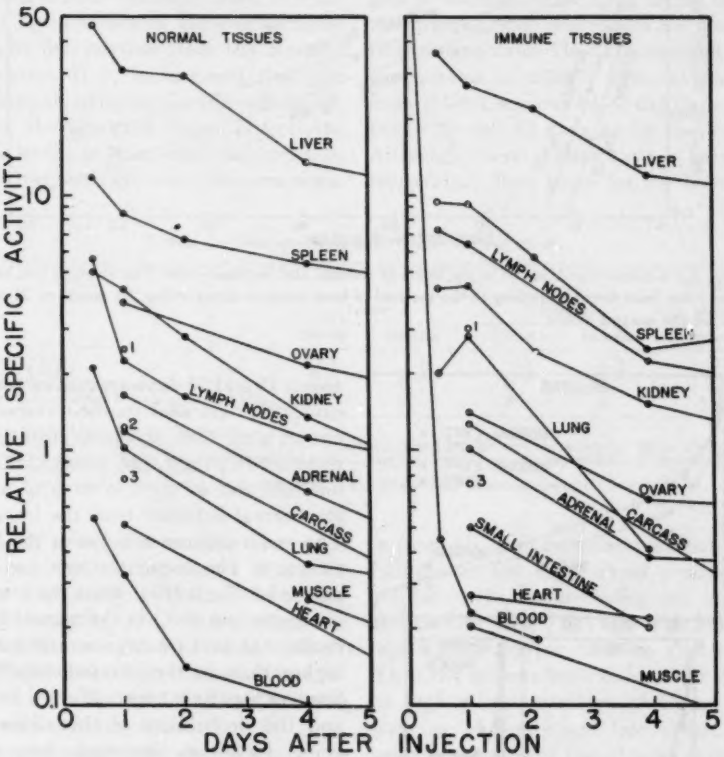

FIG. 2.—Concentration of S^{35} in the tissues of normal and immune mice. Relative specific activity equals percent of the administered S^{35} per g dry weight of tissue. This figure includes data for all the tissues analyzed from the second experiment during the first 5 days, except the brains. At one and 4 days, respectively, the normal brains were 0.025 and 0.025 while the immune were 0.10 and 0.074. The carcasses are from the first experiment. Indicated by one point only are (1) bone marrow, (2) small intestine from normal animals, (3) mesentery. The activity of blood from immune mice fell from 200 to 100 during the first hour after injection.

Liver.—The difference in concentration of S^{35} in normal as compared to immune livers shown in figure 3 is of high statistical significance. If the individual points for the 4 pairs of observations from the second experiment be-

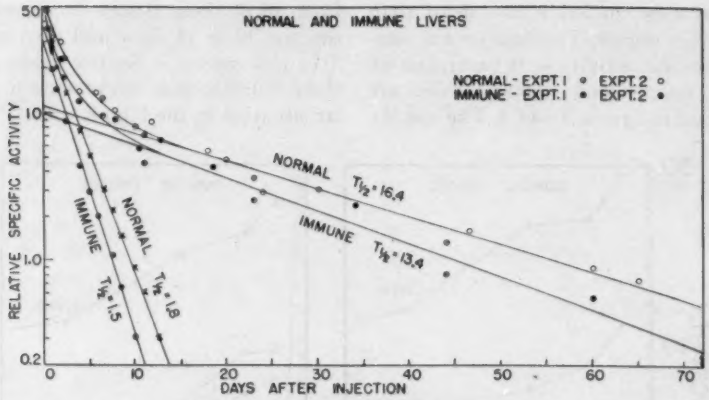

FIG. 3.—Concentration of S^{35} in the livers of normal and immune mice. The straight line beyond 15 days has been fitted according to the method of least squares disregarding the points at 24 and 72 days for the normal livers.

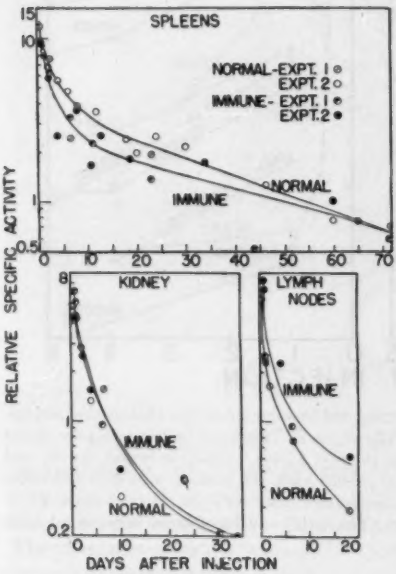

FIG. 4.—Concentration of S^{35} in the spleens, lymph nodes, and kidneys of normal and immune mice. The normal spleens at 44 days were 0.35. The points at 44 days were disregarded in the least squares calculation for the data between 15 and 72 days. These curves were all fitted by inspection.

tween 10 and 35 days are moved toward each other parallel to their respective curves until their abscissae correspond, there are 16 paired observations between one-half and 60 days in each of which the normal is higher than the immune. The mean difference between the logarithms of the concentrations for each pair is 0.107 ± 0.0134. Thus the average concentration of S^{35} in the normal livers during the first 60 days was $28 \pm 3.1\%$ higher than in the immune. Applying Student's paired t test, $N = 15$, $t = 8.0$, and the probability of this difference being fortuitous is much less than 0.00005. This is clearly a minimum estimate of the difference which might be found if the most highly immune mice only were used and the optimal dose, route and timing of the antigen injection were known. It may be seen from figure 3 that this normal-immune difference was already established by the end of 12 hours and was less than doubled during the next 60 days.

The rate of decrease of radioactivity in both normal and immune livers becomes almost constant after 12 or 15

days, and the resemblance of the curves of figure 3 to the composite curves obtained for the decay of mixtures of independent radioactivities of different half-lives suggested that they might be resolved into two straight lines, as indicated, by subtracting the extrapolated straight line portion from the remaining curve. If it is assumed that the resulting straight lines have a real significance, the observed decay of activity in the livers, at least after one day, may tentatively be ascribed to two processes, rather than normal radioactive metabolites will be discussed later.

Spleen.—Data for the spleens graphed in figure 4 are less consistent than those for the livers. Although the normal groups average about 10% higher than the immune, the difference is not statistically significant. The data reported for the spleens in table 2 were calculated from a least squares fit of the data between 15 and 72 days as for the liver. Although these results have a greater uncertainty than those for the liver, it

TABLE 2.—*Disappearance of S^{35} from liver and spleen.*

	SAγG				P^{32}-TMV*
	Livers		Spleens		Livers
	Normal	Immune	Normal	Immune	Immune
Short T½ days	1.8	1.5	1.8	1.8	2†
Long T½ days‡	16.4	13.4	27.3	41.2	12
Start of straight line days	15	12	17	18	14
% of 24-hr. activity at start of straight line	19	22	30	20	3–4

* Data reported by Libby and Madison.[3]
† This is the value obtained directly from the curve of activity versus time, as were the values for SAγG. From their mathematical treatment Salley and Libby concluded that 1.5 days is probably a better approximation of the true half-life of tobacco mosaic virus in the mouse.
‡ From least squaree calculation for the data between 15 and 72 days after injection (see figs. 3 and 4).

one with a half-life of about 1.6 days and one with a half-life of about 15 days. The values obtained from the graph together with similar data for the spleens are given in table 2. Data for P^{32}-labeled tobacco mosaic virus reported by Libby and Madison[2] are also included for comparison.

It can be seen from the data given in table 2 that 20% or more of the 24-hour activity from S^{35}AγG in the livers and spleens remained at the start of the straight line decay to be disposed of by this slower process. After 200 days one group of 5 normal mice had relative specific activities of 0.20 in the liver and 0.36 in the spleen. This is about 1% and 4%, respectively, of the activity present at 24 hours. Reasons for believing that this slow process, as well as the more rapid one, is concerned with the elimination of antigenic material is probably safe to conclude that the half-life of the more rapid process is similar to the corresponding one in the liver, while that of the slower process is significantly longer than in the liver. Thus the spleens have only about 30% as high a concentration of S^{35} as the liver at 24 hours and lose about the same proportion of the 24-hour activity during the first 15 days, but after 60 days they have about the same concentration as the liver.

Some of the factors contributing to the variability of the data from the spleen may be associated with the following findings: The spleen was significantly enlarged and variable in size after both single and multiple injections of SAγG and splenomegaly was accompanied by a decrease in the concentration of S^{35}. The spleen is characteristically prone to enlarge in response to inter-

current bacterial infections. The low points for the normal spleens at 18 and 20 days in the second experiment occurred simultaneously with an outbreak of *Salmonella enteriditis* infection which caused the death of several mice. The specific activity in the liver and spleen of one of the sick mice was only 50% and 30%, respectively, of that in apparently uninfected mice. Thus it is likely that the low point on the normal liver curve at 24 days may have been the result of this same infection. It is hoped that such disturbing factors may eventually be controlled in order to obtain more satisfactory data for the spleen, since it is probably at least as important as the liver in antibody synthesis. (For references and some recent observations see W. H. and L. G. Taliaferro.)[8]

Lymph Nodes.—The higher concentration of radioactivity in immune than in normal lymph nodes (fig. 4) may be related to the binding of antigen by antibody in these organs. Evidence for the formation and storage of antibodies in lymph nodes and lymphocytes has been presented by McMaster and Hudack[9] and others (reviewed by Taliaferro[10]).

Kidney.—The absence of any consistent normal-immune difference in the kidney (fig. 4) may be due to the scarcity of lymphoid-macrophage elements

FIG. 5.—Section of spleen (10 μ thick) from normal mouse 48 hours after injection of 1 mg of $S^{35}AγG$ containing ca 2 μc S^{35}. At the time exposure was started, 2 weeks after injection of the mouse, similar sections gave ca 44 c.p.m./cm^2 in the internal counter used in this work.

FIG. 6.—Radioautograph of section from figure 6 on Eastman no-screen medical X-ray film exposed for 30 days. Darkening is due to S^{35} activity; control sections gave no visible darkening.

8. Taliaferro, W. H. and Taliaferro, L. G. 1950, J. Infect. Dis. **87**: 37–62.
9. McMaster, P. D. and Hudack, S. S. 1935, J. Exper. Med. **61**: 783–805.
10. Taliaferro, W. H. 1949, Ann. Rev. Microbiol. **3**: 159–189.

and the lack of importance of the kidney in the synthesis of blood protein.

Localization of S^{35} within tissues and cells

Contact radioautographs were made of Zenker-formol-fixed and paraffin-imbedded unstained sections* of liver and spleen by clamping slides bearing the tissues against Eastman No Screen X-ray film. Control sections prepared in the same manner from normal animals were always mounted on each slide with the sections from $S^{35}A\gamma G$-injected animals. Figures 5 and 6 show a representative photomicrograph and radioautograph of a section of spleen from a mouse autopsied 48 hours after an intravenous injection of 1 mg of $S^{35}A\gamma G$. The exposed portions of the radioautograph correspond to the red pulp of the spleen. Hence S^{35} was concentrated in the area of the Billroth cords and venous sinuses where slow-moving blood is in direct contact with large numbers of macrophages. Autographs from the liver, on the other hand, showed a uniform distribution of radioactivity within the limits of resolution of this film. In comparing the concentrations of S^{35} in the liver and spleen, therefore, it is probably more significant to consider only the red pulp of the spleen. Since the red pulp is roughly half of the volume of this spleen, its relative specific activity is probably nearer to 16.6 than to the average 8.3 obtained from analysis of the whole organ (table 1). On this basis the concentration in the part of the spleen active in storing antigen may be 50% of that in the liver at one day and up to twice that in the liver by 50 to 60 days. Of course any such data provide only a minimum estimate of the possible concentration of antigen at the site where it actually exerts its influence on the formation of antibody. For instance a preliminary investigation of the distribution of S^{35} within liver cells with repect to the morphological fractions obtained by Hogeboom et al[11] showed that the specific activity of the mitochondria was 1.6 times that of the whole liver on a dry weight basis.

Identification of S^{35}-containing compounds in the tissues

Specific precipitation with antiserum in the presence of carrier antigen was studied as a possible method to determine whether the activity present in the liver after the onset of a linear logarithmic decay might be associated with antigen or high molecular weight antigen fragments. The results of these investigations are summarized in the diagram shown at the top of the next page.

The $S^{35}A\gamma G$ had been stored in the refrigerator for 4 months and, assuming a half-life of 300 days as shown in paper I,[8] it was estimated to have 30% of its S^{35} in dialyzable form. Crystallized salt-free ovalbumin (OV) was obtained from Armour and Company. Anti-OV rabbit serum with optimal proportion ratio of 1000 (1 ml serum flocculated fastest with 1.00 mg OV) and anti-γG serum with an optimal proportion of 600 (1 ml serum flocculated fastest with 1.67 mg $SA\gamma G$) against the $SA\gamma G$ were supplied by Dr. Moulder. Anti-γG serum rather than anti-$SA\gamma G$ was used to avoid the possibility of low molecular weight compounds containing SA being bound specifically in the precipitates.

For the experiments with liver homogenates the livers were chilled im-

* The author wishes to express appreciation to Dr. R. M. Lewert of the Department of Bacteriology and Parasitology for his assistance with the histological work.

11. Hogeboom, G. H., Schneider, W. C. and Pallade, G. E. 1948, J. Biol. Chem. 172: 619–635.

PRECIPITATION OF SAγG WITH ANTI-γG SERUM IN SUPERNATANTS FROM MOUSE LIVER HOMOGENATES

A) *Completely defined in vitro system*

Ovalbumin (OV) and $S^{35}A\gamma G$

+ anti-OV	+ anti-γG
↓	↓
precipitate	precipitate
0.1% of $S^{35}A\gamma G$	74% of $S^{35}A\gamma G$

B) *Supernatants from mouse liver homogenates*

I. Normal mice

homogenate supernatant plus OV and $S^{35}A\gamma G$

+ anti-OV	+ anti-γG
↓	↓
precipitate	precipitate
0.1% of $S^{35}A\gamma G$	16% of $S^{35}A\gamma G$

II. Mice injected with $S^{35}A\gamma G$ (34 hours)

homogenate supernatant plus OV and $SA\gamma G$

+ anti-OV	+ anti-γG
↓	↓
precipitate	precipitate
0.1% of S^{35}	0.3% of S^{35}

mediately after autopsy, frozen at −25 C for 12 hours, thawed and refrozen twice, homogenized in 2 volumes of cold distilled water containing 1 mg per ml of SA and sodium sulfate carrier at pH 7 and centrifuged in the cold at 18,000 *g* in a Servall model SS-1 angle centrifuge for 30 minutes. The supernatants were recentrifuged until about 50% of the volume of the original homogenate was obtained as a clear solution. These supernatants, adjusted to a salt concentration of 0.9% with concentrated phosphate-saline, pH 7.4, remained clear for at least a week at 0 to 3 C, but they became cloudy in 20 to 30 minutes at 37 C and formed a precipitate amounting to 10 mg dry wt/ml after one hour. This precipitate from normal livers did not adsorb $S^{35}A$ or $S^{35}O_4^=$ but firmly bound up to 25% of 0.15 mg/ml of $S^{35}A\gamma G$. Therefore the immune precipitations were started for 20 to 30 minutes at 20 to 25 C and then refrigerated for 24 to 48 hours. Control supernatants remained clear under these conditions.

A preliminary experiment in a completely defined system indicated that neither $S^{35}A\gamma G$ nor low molecular weight products of its disintegration were nonspecifically adsorbed on a precipitate formed in their presence, but that at least 68% of the intact $S^{35}A\gamma G$ present was precipitated by anti-γG serum. The system consisted of 1 mg OV plus 1.6 mg $S^{35}A\gamma G$ in 9 ml of phosphate saline, pH 7.4 (0.01 *M* buffer in 0.85% sodium chloride) plus 1 ml of carrier $SA\text{-}SO_4^=$ (10 mg SA, 20 mg sodium sulfate per ml adjusted to pH 7). One ml of anti-OV serum was added and after 20 minutes at 37 C, the solution was refrigerated for 2 hours. The precipitate was washed 3 times in the cold with 2 to 3 ml of phosphate-saline, suspended in 1 ml of distilled water and plated for counting. One ml of anti-γG serum was added to the supernatant, and the resulting precipitate was prepared for counting as above. The precipitate with anti-OV contained ca 0.07% (10 CPM) of the total S^{35}. The anti-γG precipitate contained 52% (8000 CPM) of the total S^{35} which represented ca 74% of the intact $S^{35}A\gamma G$.

In a similar experiment in which the supernatant of a normal liver homogenate was substituted for the phosphate saline the anti-OV precipitate contained about .06% of the S^{35} and the anti-γG precipitate about 16%. This inefficient precipitation of $S^{35}A\gamma G$ may have been

due in part to interference by the liver homogenate but the SAγG-anti-γG system itself did not precipitate very readily in the cold.

Liver homogenate supernatants from mice injected 34 hours or 17 days previously with 2 mg of S^{35}AγG were treated similarly to the normal liver homogenates, except that the SAγG added to the system was nonradioactive. Although the anti-SAγG precipitates contained somewhat more S^{35} than the anti-OV, no precipitate contained over 0.3% of the total supernatant activity. Portions of these supernatants were dialyzed in the cold in Visking casings against three successive portions of 200 volumes each of phosphate-saline, pH 7.4, for a total of 19 hours with stirring. Under these conditions, 35% of the S^{35} of the 34-hour supernatant and 79% of that of the 17-day supernatant was nondialyzable. It thus appears that a large portion of the S^{35} remaining in the liver for 17 days is bound to material which is not free intact SAγG but which has a molecular weight too large to allow it to pass through the wall of Visking tubing and too small to allow it to sediment in a centrifugal field of 18,000 g. Moreover, the proportion of the total S^{35} bound in this way apparently increases between 1 and 17 days.

DISCUSSION

Results obtained in vivo with S^{35}AγG indicate the potential usefulness of this substance for studies on the metabolism of an antigen. Especially important are the absence of accumulation of radioactivity in the carcass which was observed in the experiments of Libby and Madison[3] and the absence of any considerable amount of free or etherial $S^{35}O_4^=$ in the urine indicating that there is probably no significant incorporation of S^{35} from the antigen into normally occurring sulfur compounds of the tissues. Finally, the half-life of S^{35} is long enough so that it was possible to trace this antigen for 200 days after injecting only about 3 μc per mouse.

The distribution of SAγG in various tissues is in accord with that of a variety of antigens as outlined in the introduction to the previous paper.[3] Examination of figure 2 shows that liver, spleen, ovary, lymph nodes, adrenals and probably the bone marrow all have higher relative specific activities than the carcass at both 1 and 4 days. With the exception of the kidney (in which the activity is presumably due simply to its excretory function) and possibly the ovary, all these organs are rich in lymphoid-macrophage elements and are active in removing foreign antigenic material from the blood stream.[10]

The liver and possibly the spleen in normal mice have been found to retain a significantly higher fraction of an injection of antigen than they do in immunized mice. Since most of this difference arises in less than 12 hours, it must be due chiefly to differences in the animals at the time of receiving the antigen rather than to long term differences in the metabolism of the antigen. The retention of antigen in most of the normal tissues is either approximately the same or higher than that in the immune. However the lymph nodes and possibly the lungs of immune animals contained considerably more antigen than those of normal ones. The results for the lung are consistent with those obtained by Warren and Dixon[12] using an antigen labeled with I^{131} to study anaphylactic shock in the guinea pig. As mentioned before, the high value in immune lymph nodes may be due to binding of antigen by antibody stored in the lymphocytes.

12. Warren, S. and Dixon, F. J. 1948, Am. J. M. Sc. **216**: 136–145.

Results from the study of the radioactivity in supernatants of liver homogenates may indicate that antigen is quickly bound in the tissues and then persists essentially intact in the liver and spleen for at least 70 days and possibly much longer. Thus antigen cannot be precipitated by homologous antiserum in supernatants from the livers of injected mice 34 hours after injection, but at least 70% of the S^{35} activity still present at 17 days is nondialyzable. In addition, approximately 20% or more of the activity found at 24 hours is still present in the liver and spleen 15 days later at the onset of a linear decay which appears to continue uninterruptedly to at least 65 or 70 days.

A somewhat similar linear decay starting after about 14 days and accounting for 3 to 4% of the 24-hour activity from P^{32}-labeled tobacco mosaic virus was assumed by Libby and Madison[2] to represent simply the rate of elimination of P^{32} which had been incorporated into naturally occurring phosphorus compounds of the liver. However, in view of the demonstrated in vivo stability of sulfanilic acid and the relatively small importance of sulfur compounds in normal metabolism, it does not seem likely that a similar explanation could hold for as much as 20% of the 24-hour activity from $S^{35}A\gamma G$. Radioactive sulfur administered as sulfate has not been found to be incorporated into protein to a detectable extent,[13,14] and sulfide sulfur is a very inefficient precursor of protein sulfur.[15,16] In the present work sulfanilic acid was found to be eliminated many times as fast as sulfate. This is in sharp contrast to the rapid incorporation and retention of radioactive phosphate in metabolic compounds.[17,18] Data represented by Lawrence and Scott[19] indicate that when radioactive phosphate was injected into normal mice, the relative specific activity of the liver 5 days after injection was about 100 times that found in the experiment with $S^{35}O_4^{=}$ reported in the present work.

Provided a substantial fraction of the S^{35} remaining in the liver and spleen after the onset of a linear logarithmic decay is associated with antigenic material, it is apparent that the elimination of this antigen does not occur according to a single, continuous first order process as was suggested for tobacco mosaic virus by Libby and Madison.[2] Thus the highest concentration of antigen in the liver and spleen is reached in the first few hours, and the very rapid initial rate of elimination decreases quickly until after about 15 days a much slower constant rate is obtained. The change in the rate of elimination of antigen from the liver and spleen as well as the difference between normal and immune animals might be considered to indicate the existence of specific binding sites for antigen which (1) would prevent complete mixing of previously injected with newly injected antigen and (2) would take up antigen less "avidly" when partially saturated. Then the process with a half-life of 15 days would be the elimination of this bound antigen and that

13. Dziewiatkowski, D. D. 1949, J. Biol. Chem. **178**: 197–202.
14. Tarver, H. and Schmidt, C. L. A. 1939, J. Biol. Chem. **130**: 67–80.
15. Dziewiatkowski, D. D. 1945, J. Biol. Chem. **161**: 723–729.
16. Dziewiatkowski, D. D. 1946, J. Biol. Chem. **164**: 165–171.
17. Hevesy, G., in Lawrence, J. H. and Hamilton, J. G. 1948, Advances in biological and medical physics, **1**: 409–450.
18. Chaikoff, I. L. and Zilversmit, D. B., in Lawrence, J. H. and Hamilton, J. G. 1948, Advances in biological and medical physics, **1**: 321–352.
19. Lawrence, J. H. and Scott, K. G. 1939, Proc. Soc. Exper. Biol. & Med. **40**: 694–696.

with a half-life of 1.5 days the elimination of free antigen. In immune animals a smaller proportion of the radioactive antigen would be bound. According to the theory of Burnet[20] it would be predicted that the antigen might be eliminated more rapidly from immune animals because of the greater efficiency of their enzymes in breaking it down. However, the most obvious difference between normal and immune animals is the presence of antibody in the latter, and neither explanation considers the possible effects of antibody on the disposition of antigen. This must await the completion of experiments on the effect of passive immunity on the disposition of this antigen in the tissues during short time intervals.

SUMMARY

The in vivo distribution and excretion of S^{35}-sulfanilic acid-azo-bovine-γ-globulin have been studied by following the amount of S^{35} present in various organs and excreta at intervals from one-half to 200 days after intravenous injection into mice. This antigen is well suited for such work because S^{35} has a conveniently long half-life, and sulfanilic acid is apparently metabolically inert. A significantly higher fraction of antigen is retained in the livers of normal than of immune mice, while the reverse is true for the skeletal lymph nodes. Elimination from the liver and spleen probably does not occur according to a single continuous first order process. The antigen is preferentially concentrated in the mitochondria of the liver and the red pulp of the spleen. A large proportion of the S^{35} activity present in supernatants from the liver 17 days after injection is bound to material too small to sediment in a centrifugal field of 18,000 *g* but too large to pass through the wall of Visking tubing and will not precipitate with anti-γG serum. About 1% and 4% of the S^{35} present in the liver and spleen, respectively, at 24 hours after injection remained for 200 days.

20. Burnet, F. M. and Fenner, F. 1949, The Production of Antibodies, ed. 2, Monograph of the Hall Institute, Melbourne, Macmillan and Co. pp. 142.

THE EFFECT OF SPLENECTOMY ON THE COURSE OF *PLASMODIUM BERGHEI* INFECTIONS IN *MICROTUS GUENTHERI*

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The isolation of *Plasmodium berghei* from the tree rat, *Thamnomys surdaster*, by Vincke and Lips (1948) provided the first mammalian plasmodium suitable for experiments with large series of animals. The following hosts have been recorded to date as susceptible in varying degrees to *P. berghei:*

1. *Thamnomys surdaster*, the tree rat of the Belgian Congo from which the parasite was originally isolated.
2. White mice and white rats (Vincke and Lips, 1948).
3. The cotton rat (Rodhain, 1949).
4. The Syrian hamster, *Mesocricetus auratus*, and the field vole, *Microtus guentheri* (Adler, Yoeli and Zuckerman, 1950). In addition, *Meriones shawi* can be infected (unpublished results, Adler, Yoeli and Zuckerman).

The guinea pig and the rabbit were reported by Vincke and Lips (1948) to be refractory to infection.

The present paper describes the course of untreated infections of *P. berghei* in the local field vole, *M. guentheri*, and the effects of splenectomy, performed at varying periods before and after inoculation, on such infections.

MATERIALS AND METHODS

A mouse infected with *P. berghei* was obtained through the good offices of Prof. H. E. Shortt in March, 1950, and the strain has subsequently been maintained in white mice and rats by blood transfer. In the present series of experiments, 40 million parasitized rat erythrocytes obtained by cardiac puncture into 4% sodium citrate were administered intraperitoneally to each of a series of local, laboratory-bred field voles, *M. guentheri*, kindly presented to us by Prof. F. S. Bodenheimer of the Department of Entomology of the Hebrew University. Blood was taken during the acute rise of an infection in order to avoid the use of parasites possibly injured by acquired immune mechanisms developing in the donor at or after crisis. The voles were unselected as to age, size or sex.

Blood films were taken daily during initial infections and during relapses occurring spontaneously or induced by splenectomy, and two or three times a week during latent periods. All films were dry-fixed in methyl alcohol absolute and stained with Giemsa's stain.

Due to the low level of many untreated initial infections, the number of parasitized red cells per 50,000 erythrocytes was counted. Since frequent multiple infection of the red cells made it difficult to determine the exact number of parasites contained in a cell, and especially since so little pigment was produced that a large group of single merozoites was indistinguishable from a mature segmenter, we counted the number of parasitized erythrocytes rather than of separate parasites.

An infected animal in which no parasites were observed in 3 consecutive examinations was considered to have become latent.

Data thus obtained were reduced to percentages or to numbers of parasitized erythrocytes per 10,000 red cells for graphic or tabular presentation.

Splenectomies were performed with sterile precautions under ether anesthesia.

White mice inoculated with tissues from voles suspected of radical cure were studied for from 13 to 26 days before a negative diagnosis was made.

EXPERIMENTAL

1. Untreated infections of M. guentheri *with* P. berghei

Untreated initial *P. berghei* infections in *M. guentheri*, terminating either in latency or death, were studied in 37 animals. Additional information on ini-

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tial infections interrupted by splenectomy or accidental death was obtained from 12 voles.

All voles receiving standard inoculums developed patent infections after very short prepatent periods. Parasites were generally found in the peripheral blood on the day following inoculation, but occasionally their appearance was delayed for a further 24 hours, or rarely for 48 hours. In a single instance parasites were first observed on the 5th day following inoculation.

Untreated infections did not always have well-defined precritical, critical and postcritical stages. Figure 1 describes a series of representative untreated infections, arbitrarily divided into the following categories: A. mild, B. low-grade, C. severe, chronic, and D. severe, fatal.

The available number counts of the 12 infections interrupted by splenectomy or terminated by accidental death were distributed approximately as were those of the 37 cases studied throughout their entire course.

Untreated *P. berghei* infections in *M. guentheri* were relatively benign, since 78% (29 out of 37) became latent, while only 22% (8 out of 37) were fatal. It is noteworthy that the experiments were carried out in 3 groups in May, June and July, respectively, and that all of the 8 deaths observed occurred in the July experiment, as may be seen in table 1. While none of the fatal infections had peak counts lower than 21%, a high parasitemia did not preclude recovery with subsequent latency, as may be seen in figure 1, section C.

A feature noted in 7 of the 37 untreated infections was the appearance of low-grade, spontaneous relapses early in latency (cf. fig. 1, group A, infections 3 and 4). Relapses occurred between the 7th and the 13th days following the onset of latency, and a single parasite was seen on the 16th day. Parasites were observed during relapse for one to 4 days, and then again disappeared from the circulation. Spontaneous relapse counts never exceeded 0.2% and were generally far lower. The number of such relapses may actually have been somewhat higher than that recorded, since films were examined during latency not daily but 2 or 3 times a week, and transient showers of parasites may therefore have been overlooked.

TABLE 1.—*Peak parasitemias of 37 initial, untreated infections of* P. berghei *in* M. guentheri, *terminated by latency or death. The data are arranged chronologically.*

Peak % red cells parasitized	May	June	July	Total
	Infections terminated by latency			
<1	8	1	2	11
1 to 10	2	1	9	12
11 to 20			2	2
21 to 30				
31 to 40		1		1
41 to 50			1	1
51 to 60			1	1
61 to 70			1	1
				29
	Infections terminated by death			
<1				
1 to 10				
11 to 20				
21 to 30			1	1
31 to 40			1	1
41 to 50			1	1
51 to 60			3	3
61 to 70			2	2
				8

Spontaneous radical cure was observed to occur in 15 out of the 29 survivors of untreated infections following latency of from 5 to 67 days' duration. This conclusion was based on the following lines of evidence (table 2):

1. Splenectomy performed up to 5 days after the termination of patency was uniformly followed by relapse; whereas in certain of the voles splenectomized after more than 5 days of latency no relapse ensued for two weeks during which blood films were examined daily. In simian malaria Knowles and das Gupta (1934) found

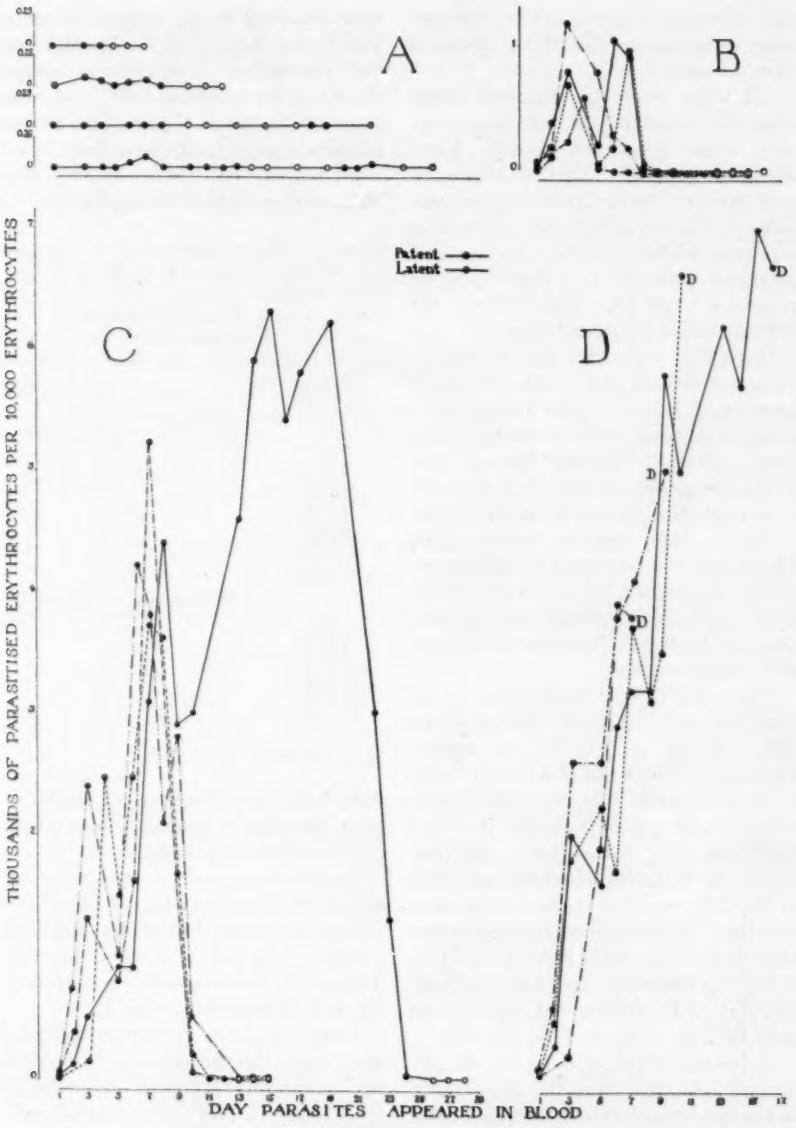

FIG. 1.—Untreated initial infections of *P. berghei* in *M. guentheri*. A: Infections with peak parasitemias below 1% (12 out of 37 animals, i.e., 32%). B: Infections with peak parasitemias between 1+ and 19% (12 out of 37 animals, i.e., 32%). C: Relatively intense but clinically mild infections with peak parasitemias 20% or above, terminating in latency (5 out of 37 animals, i.e., 14%). D: Relatively severe infections with peak parasitemias 20% or above, terminating in death (8 out of 37 animals, i.e., 22%).

splenectomy to be the method of choice in establishing latency, when other methods such as protein shock and blood transfer had failed. Absence of relapse in voles following splenectomy was thus taken as presumptive evidence of radical cure.

2. Spleens of voles suspected of cure, ground in saline and inoculated into susceptible mice failed to infect, although it is known that as few as 40 infected red cells establish rapidly fatal infections in our strain of mice.

3. Voles which failed to relapse for two weeks after splenectomy were bled by cardiac puncture, and their citrated blood was inoculated intraperitoneally into mice. (Either ground spleen or/and citrated blood from each of the voles suspected of cure was thus inoculated into mice.) None of the bloods from nonrelapsing voles was infective.

4. All surviving mice whose blood was microscopically negative for several weeks following the inoculation of vole tissues received doses of mouse blood infected with *P. berghei* 2 to 3 months later. All developed fatal infections as severe as those in clean controls infected with the same inoculum. The fact that the infections developed in all respects as if in clean mice, with no evidence of immunity to superinfection argues in favor of the noninfectivity of the microtus tissues, and thus in favor of cure of the tested voles following initial, acute infection.

TABLE 2.—*Evidence supporting spontaneous radical cure of* P. berghei *infections in* M. guentheri *at varying periods following the disappearance of microscopically demonstrable parasites from the circulation.*

Microtus number	Splenectomy			Cardiac blood inocul. into mice in which infect. failed to develop for 13 to 26 days thereafter		Days between inocul. of vole tissues into mice and challenge inoc. leading to fatal mouse infection
	Days between inoculation and splenectomy	Days since last parasite was seen in the peripheral blood	Relapse during fortnight after splenectomy	Days between inoc. of voles and card. punct.	Days since last parasite was seen in the periph. blood	
1	20	7	none	36	23	
2	24	15	none	41	32	66
3	26	13	none	49	36	
4	26	16	none	49	39	
5	26	7‡	none	49	30	
6	29	16	none	44	31	
7	29	19	none	44	34	
8	29	19	none	44	34	
9*	29†	12				78
10*	29†	5				78
11*	61	36§	none			
12*	75†	63				79
13*	75	57‖	none	91	73	79
14*	75	68	none	91	84	79
15*	75	49‖	none	91	65	79

* Spleens of these animals were inoculated into mice in which infection failed to develop for 13 to 21 days thereafter.
† Died following operation.
‡ Following 2-day spontaneous relapse.
§ Following 4-day spontaneous relapse.
‖ Following 1-day spontaneous relapse.

The evidence supporting the view that 15 of the 29 latent infections studied underwent spontaneous radical cure following a more or less protracted period of latency is summarized in table 2.

2. *Effect of splenectomy on* P. berghei *infections in* M. guentheri

The effect of splenectomy on the course of *P. berghei* infections in *M. guentheri* was studied in the following animals: 15 voles were splenectomized 2 to 16 days prior to inoculation and 35 were splenectomized at intervals 1 to 75 days after inoculation. Sple-

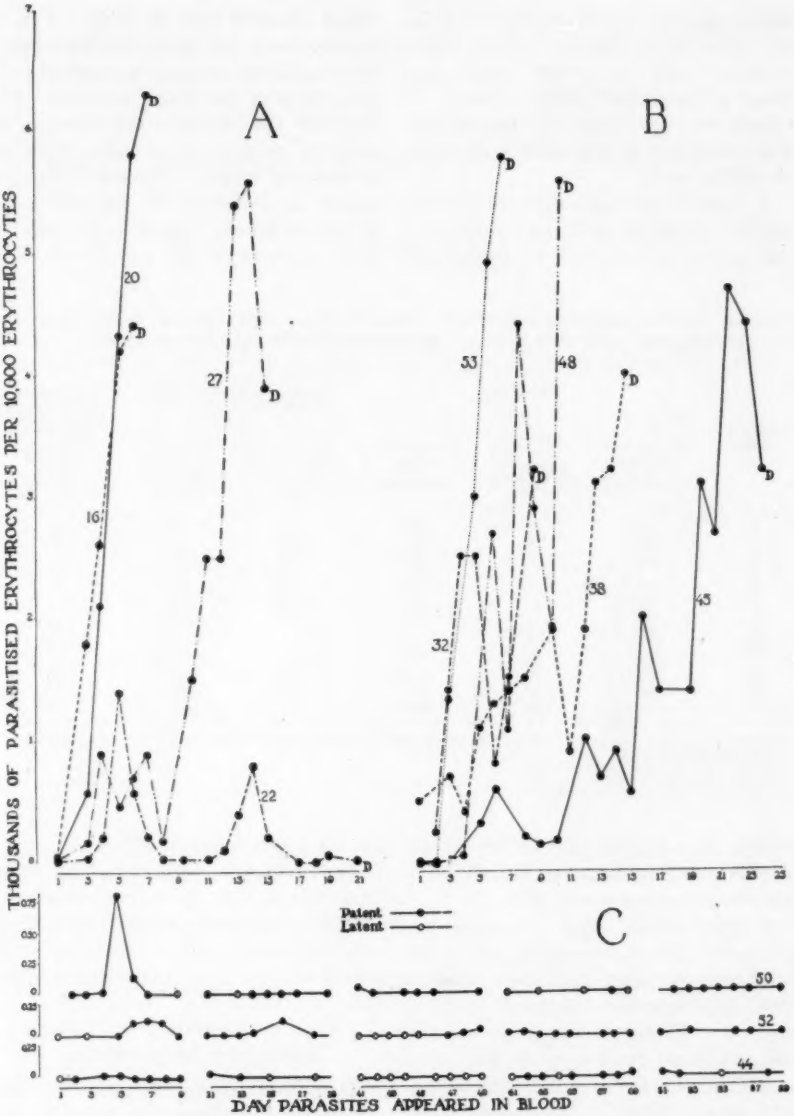

FIG. 2.—Infections of *P. berghei* in *M. guentheri* following splenectomy. A: Infections in animals splenectomized prior to the inoculation of parasites, including samples covering the entire range of severity and duration in this group. (N.B.: the abscissa represents days after inoculation when parasites appeared in the blood.) B: Fatal infections following splenectomies performed 1, 7, 14, 20 and 26 days after the inoculation of parasites. (N.B.: the abscissa represents days after splenectomy when parasites appeared in the blood.) C: Chronic infections following splenectomies performed 13, 24 and 26 days

nectomies were thus performed during the entire period covered by initial, uninterrupted infections terminating in death or in developed latency.

As with uninterrupted initial infections, splenectomized animals were studied in 3 groups during May, June and July, 1950. Since no marked differences were noted among the infections in the 3 experiments, as may be seen in table 3, the results were pooled. Table 4 presents data describing the infections of the 50 splenectomized voles studied. Figure 2 graphically depicts representative infections after splenectomy performed before inoculation (A), during patency or latency, and ending in death (B) and during latency and ending in chronic infection (C).

Relapses in group B were essentially similar to initial infections in group A, revealing no evidence of the acquisition of immunity by animals succumbing to relapse. Group C illustrates the first evidence of acquired immunity observed.

Splenectomy had a profoundly disturbing effect on the ability of voles satisfactorily to adjust to the presence of the parasite. Thus, infections were fatal in all 15 animals splenectomized prior to the inoculation of parasites (table 4, voles 16 through 30), and in all splenectomized up to 10 days after inoculation (table 4, voles 31 through 41). When splenectomy was performed between the 13th and the 26th days after inoculation, the voles responded in 3 different ways:

1. Nine out of 17 had fatal relapses essentially similar to initial infections in voles splenectomized before inoculation.

2. Three out of 17 developed low-grade, permanent, chronic infections, characterized by alternating periods of latency and patency and lasting at least 150 days.

TABLE 3.—*Peak parasitemias during the patent period following splenectomy in* 38 P. berghei *infections in* M. guentheri, *arranged chronologically. The infections terminated in death or led to a chronic, low-grade patency. Infections terminating in cure* (12 *in all*) *had no patent periods following splenectomy.*

Peak % red cells parasitized	May	June	July	Total
	Infections leading to chronic condition			
<1		1		1
1 to 10	1		1	2
				3
	Infections terminated by death			
<1				
1 to 10	1		1	2
11 to 20	1	1		2
21 to 30	2	2	2	6
31 to 40	1	9	2	12
41 to 50	2	3	2	7
51 to 60	1	1	2	4
61 to 70	1	1		2
				35

3. Five out of 17 underwent spontaneous, radical cure (cf. tables 2 and 4).

All 7 of the voles splenectomized between the 29th and the 75th days following inoculation had undergone spontaneous cure prior to the operation (cf. tables 2 and 4).

Thus, all animals splenectomized before and during patency and up to the 5th day of latency succumbed to the infection. Between the 5th and the 15th days of latency splenectomy may be followed by death, chronic infection or

after the inoculation of parasites Short, isolated periods during chronic infections are presented to illustrate their low-grade, persistent nature. (N.B.: the abscissa represents days after splenectomy when parasites appeared in the blood.) Note that no relapse followed the operation in 12 cured voles splenectomized 20 to 75 days after inoculation.

TABLE 4.—*Résumé of data on the initial infections of* P. berghei *in 15* M. guentheri *splenectomized at varying periods before inoculation and on the relapse infections of 35* M. guentheri *splenectomized at varying periods during and after acute initial infections.*

Microtus number*	Splenectomy X days before (−) or after (+) initial inoculation	Days patent before splenectomy	Days latent before splenectomy	% peak parasitemia before splenectomy	Days between splenectomy and initiation of relapse	Days patency of relapse or initial infection following splenectomy	% peak parasitemia after relapse or initial infection following splenectomy	Final outcome of infection
16	−16					7	44	death
17	−16					11	31	death
18	−16					8	43	death
19	−16					11	25	death
20	−16					8	63	death
21	− 8					10	25	death
22	− 8					21	14	death
23	− 8					10	9	death
24	− 2					10	35	death
25	− 2					8	35	death
26	− 2					13	32	death
27	− 2					15	56	death
28	− 2					8	49	death
29	− 2					9	50	death
30	− 2					7	56	death
31	+ 1	1		<1		9	29	death
32	+ 1	1		<1		9	32	death
33	+ 4	2		<1		16	44	death
34	+ 4	4		<1		12	66	death
35	+ 4	1		<1		12	35	death
36	+ 7	7		<1		16	44	death
37	+ 7	7		1		16	24	death
38	+ 7	7		7		16	40	death
39	+10	10		<1		14	39	death
40	+10	10		<1		7	47	death
41	+10	8		<1		16	42	death
42	+13		5	5	1	10	16	death
43	+13	13		1		18	35	death
44	+13		5	<1	2	186‡	1	chronic
45	+14	14		3		25	47	death
46	+14		6	2	2	28	40	death
47	+14		3	52	4	35	26	death
48	+20		7	2	2	11	56	death
1	+20		7	2				cure
49	+24		14	12	2	10	24	death
50	+24		13	42	2	142‡	8	chronic
51	+24		14	9	2	10	9	death
2	+24		15	2				cure
3	+26		12	5				cure
52	+26		15	6	5	187‡	2	chronic
53	+26		3†	<1	2	6	58	death
4	+26		17	<1				cure
5	+26		6	<1				cure
6	+29		15	20				cure
7	+29		18	10				cure
8	+29		18	11				cure
11	+61		36	37				cure
13	+75		56†	<1				cure
14	+75		67	<1				cure
15	+75		49†	<1				cure

* For no. 1 to 15 see table 2.
† Following a spontaneous relapse of several days of duration.
‡ This is a minimal number of days. The infections were still patent when the present paper went to press.

cure. After this period of latency (i.e., at least 20 days following inoculation) all splenectomized animals studied gave evidence of cure.

In general, fatal postoperative parasitemias were significantly higher than preoperative parasitemias which had previously run their entire course in the same animals.

In summary, the outcome of an infection following splenectomy was roughly a function of the time elapsing

between inoculation and splenectomy, a short interval being followed by death and progressively longer intervals being associated with death, chronic infection or cure.

3. *Analysis of the immune reactions in* P. berghei *infections in* M. guentheri *in the light of the data in parts 1 and 2*

Since low-grade infections of *M. guentheri* with *P. berghei* usually remain at a plateau level gradually descending into latency, while more intense infections take a progressive, fulminating course ending in death, no clear-cut crises can generally be defined in these infections. This fact complicates discussion of the immune reactions in terms of innate and acquired immunity, terms defined with reference to crisis. Since, however, it may be assumed that immune reactions during the first few days following inoculation of the parasites are likely to be nonspecific, whereas those observed late in patency and certainly those occurring at or just before the onset of latency must be associated with acquired immunity, immune reactions are here discussed in the classic terms, with the understanding that the transition between innate and acquired immunity cannot here be as sharply distinguished as in malarial infections characterized by well defined crises.

In attempting to analyze evidences of innate immunity to *P. berghei* in *M. guentheri* the following method was therefore adopted. The initial stage of an untreated infection was arbitrarily considered to range between the 1st and the 7th days following inoculation, since most of the infections were patent throughout this period when a standard inoculum was employed, and since the slow descent into latency rarely began before the 7th day, which was thus assumed to be the time when some acquired immunity supervenes. The total number of parasitized red cells observed during this time was computed for each animal and was expressed as parasitized red cells per 10,000 red cells over a period of 7 days, i.e., parasitized red cells per 70,000 red cells. In a sense this sum expresses the area of the geometrical figure subtended by the curve describing the initial course of the infection and bounded at the right (on the 7th day) by a perpendicular upon the abscissa, representing number of days (cf. fig. 3). The area of such a figure emphasizes total parasitemias observed, while the course of the initial rise in parasitemia, which fluctuates in individual infections, is relatively less important. Such total initial parasite counts for 48 untreated infections and for 15 infections induced following splenectomy are presented in table 5.

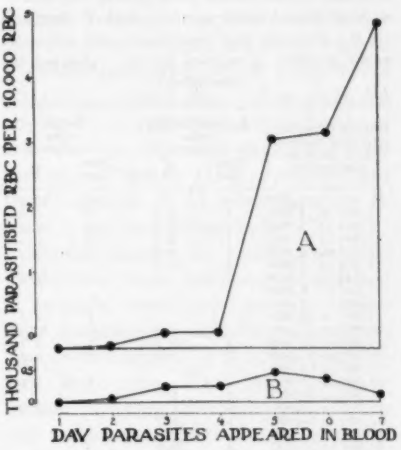

FIG. 3.—Figures illustrating the total number of parasitized erythrocytes observed during the first 7 days of infections of *P. berghei* in splenectomized (A) and intact (B) voles. Each figure represents an infection approximating the mean of its group, cf. table 5.

There is a significant difference between the means of these two groups. M-intact $= 3900 \pm 1359$; M-splenectomized $= 10{,}800 \pm 531$; and the standard error of the difference between the means $= 1458$. There are thus 4.732 such standard errors in the observed difference between the means.

TABLE 5.—*Sums of daily parasite counts in intact and splenectomized voles during the first 7 days of initial acute infection with* P. berghei. (*N.B.: Parasites were inoculated into splenectomized animals at varying periods following the operation.*)

Sum of parasitized red cells observed in 7 daily examinations	Splenectomized voles		Intact voles	
	Number	%	Number	%
<1,000			25	52.1
1,001 to 2,000	1	6.7	3	6.3
2,001 to 3,000	1	6.7	2	4.2
3,001 to 4,000	1	6.7	1	2.1
4,001 to 5,000	1	6.7	2	4.2
5,001 to 6,000	2	13.3		
6,001 to 7,000			3	6.3
7,001 to 8,000				
8,001 to 9,000				
9,001 to 10,000			2	4.2
10,001 to 11,000			5	10.4
11,001 to 12,000	2	13.3	1	2.1
12,001 to 13,000	2	13.3	2	4.2
13,001 to 14,000				
14,001 to 15,000			1	2.1
15,001 to 16,000	1	6.7		
16,001 to 17,000			1	2.1
17,001 to 18,000	1	6.7		
18,001 to 19,000				
19,001 to 20,000	3	20.0		

While the difference between the total intact and splenectomized populations is clear, about half of the intact voles developed infection within the range of those of the splenectomized animals. The presence of a relatively high degree of innate immunity to *P. berghei* is therefore reflected in the low parasitemias developing in about half of the intact voles studied. Since we have seen that *P. berghei* is able under other conditions (i.e., following splenectomy) to develop in the same animal as a fulminating infection, these low parasitemias do not represent a basic incompatability between the host and the parasite but rather indicate that some mechanism is suppressing the parasite in a relatively efficient manner in the intact voles with mild infections. Our data do not indicate whether this suppression is parasiticidal or reproduction-inhibiting. However, whatever its mode of activity, the suppression must be associated with the presence of the spleen during the period when only innate immunity is functioning, since splenectomy prior to inoculation regularly resulted in severe, fatal parasitemias during the initial period, at a time when at least half of the intact population experienced extremely mild, transitory infections.

Evidence of the acquisition of immunity during the course of infection of *M. guentheri* with *P. berghei* is seen in the low death rate, in the early onset of latency, in the development of chronic infections and in the high rate of cure.

The development of efficient acquired immunity capable of protecting the host against fatal outcome of the infection is usually delayed until some time following the onset of latency. Thus, relapses following splenectomy performed up to this time are frequently no less severe than, and as rapidly fatal as, initial infections induced following splenectomy, i.e., when acquired immunity can be ruled out (cf. fig. 2).

Thus, during patency or early latency no demonstrable protective action, such as that exercised throughout infection by the spleen, has yet been developed by any other organ, including those of the lymphoid macrophage system, nor is any protective activity associated as a humoral factor with the circulating blood during this period.

The fact that appreciable acquired immunity involving tissues other than the spleen does finally develop later in latency is demonstrated in the chronic type of infection following splenectomy, observed in three animals splenectomized 13, 24 and 26 days after inoculation, and 5, 13 and 15 days, respectively,

following the onset of latency. In these cases a lasting equilibrium was established between the parasite and the host, but tissues other than the spleen, while able to hold the parasite in check, were not capable of replacing the spleen in achieving cure even after more than 150 days, a considerable portion of the animal's life span.

The most complete demonstration of the acquisition of immunity lies in the high rate of cure observed in voles splenectomized late in latency. Not only has the immune process checked the deleterious effect of the parasite on the host, as in chronic infections, but it has eradicated the parasite completely, a condition uncommon in most malarial infections.

Our data show that both innate and acquired immunity play a role in infections of *P. berghei* in *M. guentheri*, and that the spleen is essential to both the mechanisms of innate and acquired immunity until at least 5 days following the onset of latency. By this time acquired immunity may be sufficiently advanced to safeguard the life of the uncured host following splenectomy or even radically to cure the infection.

DISCUSSION

Through the study of experimental infections of *P. berghei* in the field vole *M. guentheri* certain fundamental problems in malariology can conveniently be attacked. Radical, spontaneous cure occurred in this mammal in a high percentage of the cases studied. Cure was proven by the absence of relapse following splenectomy and by the noninfectivity of tissues from cured animals in susceptible hosts. The problem of radical cure in malaria, i.e., sterilization of the infection, is one of the aspects of malariology least systematically studied to date. Discussions of clinical cure frequently refer loosely to the absence of clinical attack. However, the term in its more restricted sense here refers to the total absence of parasites, experimentally demonstrated, and not merely to failure to discover the parasites microscopically in the peripheral blood.

In spite of its obvious importance to chemotherapy, the difficulty of accurately defining recovery in human malaria makes it practically impossible to determine whether a human patient has undergone radical cure. Thus Hackett (1937) stated that "it is virtually impossible to say when a cure has been achieved in (human) malaria"; and Stratman-Thomas (1941) stated that the "duration of an infection with *P. vivax* is not accurately known."

In our study all voles examined 29 days or more following inoculation (7 animals) were found to have undergone spontaneous radical cure, and cure was demonstrated in 5 voles even before this time. The importance of further studying the mechanism of cure and its relationship to that of acquired immunity in mammalian malaria is clear. The high rate of cure in its above defined restricted sense in *P. berghei* infections in *M. guentheri* makes this host-parasite combination a suitable test object for systematic studies of radical cure in mammalian malaria.

In general, untreated infections in the vole differ in certain additional respects from those in other laboratory hosts, such as the mouse, hamster and rat. Those in the vole tend to be mild and transient, leading to a short latency period followed by cure. In the mouse and hamster, in contrast, the infections are progressive, fulminating and almost invariably fatal. The infection in the rat is intermediate between the two above types, with a higher death rate than in the vole and with numerous protracted, chronic infections. The course

in the vole appears most closely to resemble that described by Rodhain in the cotton rat (1949).

The importance of the spleen in protecting *M. guentheri* against *P. berghei* has been clearly demonstrated. The profoundly depressing effect of splenectomy on the defenses of *M. guentheri* against *P. berghei* is in line with the results of similar studies in simian malaria. Thus Krishnan et al (1933), working with *Plasmodium inui*, showed that splenectomy increased both the severity and the mortality rate in 3 species of monkey. Malamos (1934) observed that splenectomy of *Macacus cynomologus* caused it to succumb to *Plasmodium knowlesi*, which is a nonfatal infection in the intact animal. Blockade of the reticulo-endothelial system led to a similar result. Mulligan et al (1940) found that *P. knowlesi* in *Silenus sinicus* is transformed by splenectomy from a moderate to a severe infection. *P. berghei* in the intact vole resembles the above mild simian infections, and their respective courses following splenectomy are very similar, both with reference to increased severity and to rise in mortality rate.

Rodhain (1949) found that splenectomy of the cotton rat infected with *P. berghei* transformed the relatively innocuous, transient parasitemia into a severe disease ending in death or in a chronic condition, and that splenectomy before inoculation led to death in all of his experimental animals. *P. berghei* thus behaves in a similar manner in *Microtus* and in the cotton rat both during initial infection and following splenectomy.

The time of removal of the spleen is decisive in determining the subsequent development of *P. berghei* in the vole. The spleen, if present, provided adequate defense in most cases during the first fortnight after inoculation, but on its removal the animal was apparently as unprotected approximately two weeks after infection as it had been before inoculation. During and after the third week following inoculation, however, some extrasplenic defense began to appear. Thus splenectomy done at this time led in a few cases to a chronic, low-grade parasitemia of long duration, the expression of an equilibrium attained between host and parasite. Whether this defense be cellular, humoral or both, our data are insufficient to determine. Functional increase in the activity of other organs of the lymphoid macrophage system was not, however, sufficient to replace the spleen even after a period of at least 150 days.

The most efficient defense against *P. berghei* is seen in the high percentage of untreated, latent infections achieving radical cure. Cure has never been observed to occur, however, unless splenectomy was deferred until the 20th day following infection, or longer. The presence of the spleen during the first three weeks is thus in some way essential to cure. Whether the mechanism responsible for clearing the parasites from the body be actually located in the spleen and possibly qualitatively, though not quantitatively, identical with that inducing suppressive action during latency; or whether the spleen simply keeps the host alive by suppressing the parasites to a minimal number during latency while the final curative mechanism is being built up elsewhere is not shown by our data.

In general splenectomy seems to have a less deleterious effect on host defenses in avian than in mammalian malarias. Thus Causey (1939) claimed that it had no demonstrable influence on *Plasmodium cathemerium* or *Plasmodium relictum* in canaries, although it did induce relapse in three out of three chronic birds. However, partial incapacitation

of the reticulo-endothelial system (Kritschewski and Demidowa, 1934) reduced the protective action of this system against *Plasmodium praecox* in the finch. By implication, the spleen was, of course, included with other tissues containing concentrations of cells of the reticulo-endothelial system. Similarly, Gingrich (1941) observed that blockade increased the severity of *P. cathemerium* in canaries, and stressed the fact that acquired and not innate immune mechanisms were involved. Terzian (1946) found that splenectomy prior to inoculation had no effect on *P. lophurae* or *P. gallinaceum* in chickens; but that during latency it induced relapse of *P. gallinaceum* though not of *Plasmodium lophurae*. Thus the spleen was associated with acquired immunity in one of the two chicken malarias but not with innate immunity in either case. Taliaferro and his co-workers (1948, 1948, 1949) found that splenectomy of chickens with *P. gallinaceum* reduced acquired immunity without affecting innate immunity.

This does not appear to hold in the case of the mammalian malarias so far studied, in which both innate and acquired immune mechanisms are adversely affected by splenectomy. Krishnan et al (1933) found that splenectomy in simian malaria resulted in a failure of natural was well as of acquired immunity. Malamos (1934) and Nauck and Malamos (1935) found that both innate and acquired immunity to *P. knowlesi* in *M. cynomolgus* are broken down by splenectomy and/or blockade. Our results with *P. berghei* in *M. guentheri* support this view, since early initial infections in intact animals, taken as a group, were lower than those in animals splenectomized before inoculation. Splenectomies done early in the infection, before acquired immunity is superimposed on innate immunity, were not included in our analysis, as no sharp crisis marks the initiation of acquired immunity, and such a point would therefore have had to be arbitrarily chosen. It is, however, noteworthy that our data on splenectomy during early initial infections support the conclusions based exclusively on splenectomy prior to inoculation.

Efficient acquired immunity, although its development is deferred until advanced latency, constitutes a very potent anti-malarial mechanism in *P. berghei* infections in the vole, since it was responsible for the radical cure of all of the infections studied during this period.

Our preliminary description of infections of *P. berghei* in *M. guentheri* (Adler et al, 1950) requires slight modification in view of the additional material obtained during the final period of this work. Whereas the infection in the vole is, indeed, relatively mild and transient, a certain number of severe parasitemias may occur, and these may be fatal. The reason for the fact that all the observed fatalities fell in July is not clear to us. Since all voles were infected with a standard number of parasitized rat erythrocytes, it was not successive passage through voles which increased the severity. No similar rise in severity was observed among the rat donors. Marked climatic fluctuations characterize the period involved, and these may have played a role in inducing the observed shift in pathogenicity.

SUMMARY AND CONCLUSIONS

Acute initial infections of *Plasmodium berghei* in the field vole, *Microtus guentheri*, are characterized by a patent period lasting for about a fortnight, followed in the majority of cases by a period of latency, occasionally interrupted by short, low-grade, spontaneous relapses. The mortality rate is 22%. In most

cases parasitemias are relatively low, with a mean peak count of about 10% in voles in which the infection becomes latent and of about 50% in those with fatal infections. A few cases of parasitemias as high as 70% have been recorded. During the 4th week after inoculation and subsequently, surviving, untreated voles undergo spontaneous, radical cure.

Splenectomy profoundly depresses the defenses of *M. guentheri* against *P. berghei*. All animals splenectomized before and during the first two weeks following inoculation died with parasitemias averaging about 40%. During the 3rd and 4th weeks splenectomy led either to death, as above, or to a chronic, low-grade infection of long duration; or it was followed by no demonstrable relapse, in all of which cases cure was demonstrated by the subinoculation of suspected tissues to susceptible hosts.

The spleen is therefore essential in establishing immunity against, and eventually in curing *P. berghei* infections in *M. guentheri*, and its removal adversely affects both the innate and the acquired immune mechanisms of the host.

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THE ROLE OF THE SPLEEN IN HEMOLYSIN PRODUCTION IN RABBITS RECEIVING MULTIPLE ANTIGEN INJECTIONS

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In a previous paper, we (1950) presented evidence that the spleen forms most of the antibody during the initial antibody rise in rabbits following a single injection of sheep red cells and then probably stops forming antibody at or near the peak serum titer. Splenic antibody then disappears at a constant logarithmic rate due to normal metabolic degradation until a constant or slowly declining level is reached which represents the continuing antibody production of nonsplenic sources. In the present study, our methods have been extended to splenectomized and intact rabbits receiving multiple antigen injections. Under these circumstances, not only is the spleen relatively less important, but hemolysin titers rise much higher in splenectomized than in intact animals. This work has been published in preliminary form (1951 and 1951b).

MATERIALS AND METHODS

Similar materials and methods were used in this study as were used by us previously (1950). Suffice it to repeat here the following salient items: The antigen consisted exclusively of sheep erythrocytes. Red cells for all injections were standardized with a Klett-Summerson photoelectric colorimeter by determining the hemoglobin released by distilled water hemolysis. For convenience, the three photometrically standardized red cell suspensions injected into rabbits may be designated approximately as small (1.25 ml of a 0.1% suspension or 2×10^7 cells), intermediate (1.25 ml of a 1% suspension or 2×10^8 cells) and large (1 ml of a 10% suspension or 1.6×10^9 cells), respectively. These amounts were always given per kg body weight of rabbit and were injected into an ear vein. The red cells for injecting and testing were from the same sheep in series 2, 10, 11 and 5 through 8. Blood from the rabbits for titrations were usually obtained daily for a week or 10 days after the initial injection and 3 times a week thereafter. Bleedings were generally discontinued after 2 to 4 months but were continued for 6 months in a few of the rabbits in series 3, 5 and 6.

All hemolysin titers are given in 50% hemolytic units and are defined as the amount of serum which produces 50% hemolysis of 1 ml of 2% sheep cells (standardized photometrically) in the presence of four 50% units of complement. The titers were read from a plot, according to von Krogh's equation, of the amount of serum against the degree of hemolysis determined photometrically. In the semilogarithmic graphs such titers are expressed directly in ml. In figure 1, table 1 and the text, they are expressed as the reciprocal of titer, the dilution of serum contained in 1 ml or the number of hemolytic units per ml. The last three methods obviously give identical figures.

In our first paper, we emphasized that the rise and decline of serum antibody followed a series of discontinuous semilogarithmic curves described by the first order equation:

$$A_t = A_0 e^{kt} \tag{1}$$

in which A_t and A_0 represent the antibody titers at times t and 0, respectively, and k represents the constant of increment. In the present work, we have fitted the data by visual inspection. This procedure indicates general trends in antibody accumulation and allows comparisons with our previous work. Fitted titers and fitted peak titers were obtained from these lines and are used in table 1. They were only slightly different but are probably more reliable than actual titers. Declines in hemolysin titer are given as the apparent half life ($t_{\frac{1}{2}}=0.693/k$). This procedure facilitates comparisons of rates of decline, although values probably only approached the true half life of antibody during the primary,

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most rapid decline after peak titer in some rabbits, as previously pointed out by us (1950). A few titers were out of line, such as the one on the 45th day in figure 2A, and a few, such as the antibody rise in figure 5B from the 7th to 16th day, fitted sigmoid curves. Few sigmoid curves were actually found, however, when closely spaced determinations were made.

Splenectomy in series 2, 6 and 8 and removal of the appendix, one kidney or one testis via laparotomy in series 9 through 11 were performed the day before or within several hours of the initial injection of antigen. In series 4, splenectomy was performed similarly in half of the rabbits and in the other half, from 14 days before the initial injection to 4 days after it. For convenience, the day of the initial injection will be considered the zero day, and all times will refer to days after the initial injection of antigen, even in the repeatedly injected series, unless expressly stated otherwise.

EXPERIMENTAL RESULTS

Four experiments, each consisting of intact (series 1, 3, 5 and 7) and splenectomized (series 2, 4, 6 and 8) groups of rabbits, were carried out to test hemolysin production after a single injection of the small or intermediate (series 1 and 2) and large (series 3 and 4) amounts of antigen and after repeated injections of the intermediate (series 5 and 6) and large (series 7 and 8) amounts of antigen. The first 4 series form a baseline for the last 4. Partial data from some of the rabbits in series 1, 3 and 4 have already been reported by us (1950) under identical rabbit numbers in the range between 42 and 112. The rabbits usually weighed between 2 and 2.5 kg when first injected.

Experiment 5 consisted of operative controls for the single large injection of antigen (series 9) and for the multiple injections of the intermediate (series 10) and large (series 11) amounts of antigen. Partial data from the rabbits in series 9 may be found in our earlier paper (1950).

Table 1A gives mean titers for all the rabbits in each of the 11 series at 2-day intervals for 16 days after the first injection of antigen. These titers are given in terms of fitted titers, as previously defined, and are oriented with respect to the first antigen injection. Within the period, peak titers occurred in the individual rabbits of series 1 through 4, 9 and 11 from the 6th to the 16th day and had not been reached in many of the rabbits of series 5 through 8 and 10. Table 1, therefore, also gives the mean peak titer for each series and the mean day on which it occurred. These data are graphed arithmetically in figure 1 from all the rabbits in series 6, 10 and 11 and from 5 typical rabbits in the rest of the series. Data in figures 2 through 7 are graphed semilogarithmically to show rates of increase and decrease. Similarities and differences between paired series are gauged throughout this paper by differences and ratios between daily titers and peak titer. These values, although obtainable from table 1, section A, are given in sections B and C to facilitate discussion. The standard errors of the differences are also given for peak titer and the day on which it occurred. Mean values for the rate of hemolysin increase of the primary rise and for the apparent half life of hemolysin during the most rapid decline are given in tables 2 and 3, respectively.

Experiment 1.—A single injection of 1.25 ml of 0.1% or of 1% sheep red cells/kg rabbit into intact and splenectomized rabbits (series 1 and 2)

All but 3 intact and 3 splenectomized rabbits in this experiment received the intermediate amount of antigen. This will be the amount usually designated hereafter, not only because the majority of rabbits received this amount but because the results from this and the small amount were earlier (1950) found to be indistinguishable. Peak titers in the intact animals were reached sooner and

were about 9 times higher than in the splenectomized ones (cf series 1 and 2 in fig. 1 and table 1).

Series 1 (21 intact rabbits).—Representative peak hemolysin titers for the intact rabbits of series 1 may be found in figure 1. Peak titers were reached between the 6th and 10th day and varied from 1:530 to 1:7500. The mean was 1:2800 ± 400. As previously reported by us (1950) after these amounts of red cells, peak titer was reached at one or two, rarely three, successively decreasing rates with *k* of the final rate approximating or equalling 0 (similar to the repeatedly injected rabbits in fig. 5). The first most rapid mean rate of increase was 1.4 ± 0.05 (table 2). After the peak, the titer declined for one to two weeks at a more or less constant and rapid rate in a given rabbit with an apparent half life of 3 to 9 days (mean of 5.5 ± 0.3 days). Thereafter, the rate of decline successively lessened until it finally approximated a plateau. The mean increase and decrease in hemolysin for 16 days are shown in table 1A.

Series 2 (7 splenectomized rabbits).—

TABLE 1.—*Mean values† for each of the 11 series of rabbits as follows: (A) hemolysin titer at 2 to 16 days, peak titer and day of peak titer with (B) differences and (C) ratios between these values.*

Series	Day after initial injection of sheep red cells								Peak titer‡	Day of peak‡
	2	4	6	8	10	12	14	16		
A. Hemolytic units per ml serum ×10³										
1	0.016	0.07	1.36	2.53	2.21	1.85	1.53	1.24	2.8 ± 0.4	7.2±0.2
2 Spl	0.01	0.01	0.04	0.2	0.24	0.22	0.20	0.18	0.3 ± 0.07	9.5±0.7
3	0.022	0.18	1.92	2.27	2.22	2.02	1.74	1.47	2.9 ± 0.3	7.8±0.4
4 Spl	0.021	0.02	0.13	0.42	0.64	0.61	0.59	0.55	0.7 ± 0.2	9.5±0.6
5	0.008	0.08	1.43	2.42	3.19	4.94	6.48	7.41	8.4 ± 1.7	18.1±2.0
6 Spl	0.017	0.01	0.02	0.032	0.15	1.70	3.43	6.07	22.2 ± 5.7	25.2±2.6
7	0.025	0.41	6.16§§	12.41	15.11	14.88	16.24	16.17	18.6 ± 1.9	11.8±1.4
8 Spl	0.006	0.06	0.90§§	7.13	13.60	23.50	30.58	36.38	45.0 ± 9.6	15.3±1.0
9 OpC	0.001	0.17	3.55	5.36	6.31	5.76	5.20	4.42	6.5 ± 1.0	8.4±0.7
10 OpC	0.001	0.09	2.20	3.58	4.68	9.45	13.16	13.76	21.5 ± 4.7	23.0±3.1
11 OpC	0.021	0.50	4.70§§	11.29	14.80	16.34	18.88	18.82	20.6 ± 4.0	13.4±1.1
B. Differences§										
Between intact and splenectomized										
1–2	0.006	0.06	1.32	2.33	1.97	1.63	1.33	1.06	2.50± 0.48***	−2.3±0.81**
3–4	0.001	0.16	1.79	1.85	1.58	1.41	1.15	0.92	2.23± 0.43***	−1.6±0.75*
5–6	−0.009	0.07	1.41	2.38	3.04	3.24	3.05	1.34	−13.8 ± 5.94*	−7.1±3.3
7–8	0.019	0.35	5.26	5.28	1.51	−8.62	−14.34	−20.21	−26.4 ± 9.78*	−3.6±1.7
Between intact and operative controls										
3–9	0.011	0.01	−1.63	−3.09	−4.09	−3.74	− 3.46	− 2.95	− 3.63± 1.1**	−0.9±0.84
5–10	−0.007	0.01	− .76	−1.16	−1.49	−4.50	− 5.67	− 6.34	−13.1 ± 5.0*	−4.9±3.68
7–11	−0.004	0.09	−1.45	− 1.12	−0.31	−1.46	− 2.64	− 2.65	− 2.0 ± 4.4	−1.6±1.78
Between operative controls and splenectomized										
9–4	0.01	0.03	3.42	4.94	5.67	5.15	4.61	3.89	5.9 ± 1.06***	−1.1±0.92
10–6	0.016	−0.08	2.18	3.55	4.53	7.75	9.68	7.69	− 0.7 ± 7.38	−2.2±4.04
11–8	0.015	0.44	3.79§§	4.13	1.20	−7.16	−11.70	−17.56	−24.4 ±10.39*	−1.9±1.48
C. Ratios										
Intact: splenectomized										
1:2	2	7	34	13	9	8	7.7	7	9	0.8
3:4	1	9	15	5	3.2	3.1	2.9	2.7	4.1	0.8
5:6	0.5	8	72	76	21	2.9	1.9	1.2	0.4	0.7
7:8	4	7§§	6.8	1.7	1.1	0.6	0.5	0.4	0.4	0.8
Intact: operative controls										
3:9	22	0.6	0.4	0.4	0.4	0.3	0.3	0.3	0.4	0.9
5:11	8	0.9	0.7	0.7	0.7	0.5	0.5	0.6	0.4	0.8
7:11	1	0.8	1	1	1	0.9	0.8	0.8	0.9	0.9
Operative controls: splenectomized										
9:4	0.05	8	27	13	10	9	9	8	9	0.9
10:6	1	9	220	112	30	6	4	2	0.9	0.9
11:8	1	9§§	5	2	1	0.7	0.6	0.5	0.5	0.9

† All mean values from fitted curves. Standard errors are given in some cases.
‡ The significance of the differences are indicated by the following: *, $P \leq 0.5$; **, $P \leq 0.01$; and ***, $P \leq 0.001$.
§ Minus values indicate that the splenectomized or operative control rabbits had higher titers and reached a peak titer later than intact rabbits.
§§ On the 5th day, ratios were 9 for series 7:8 and 14 for series 11:8. Titers for series 7, 8 and 11 on the 7th day were 9.53, 2.97 and 7.82 (×10³) units, respectively.

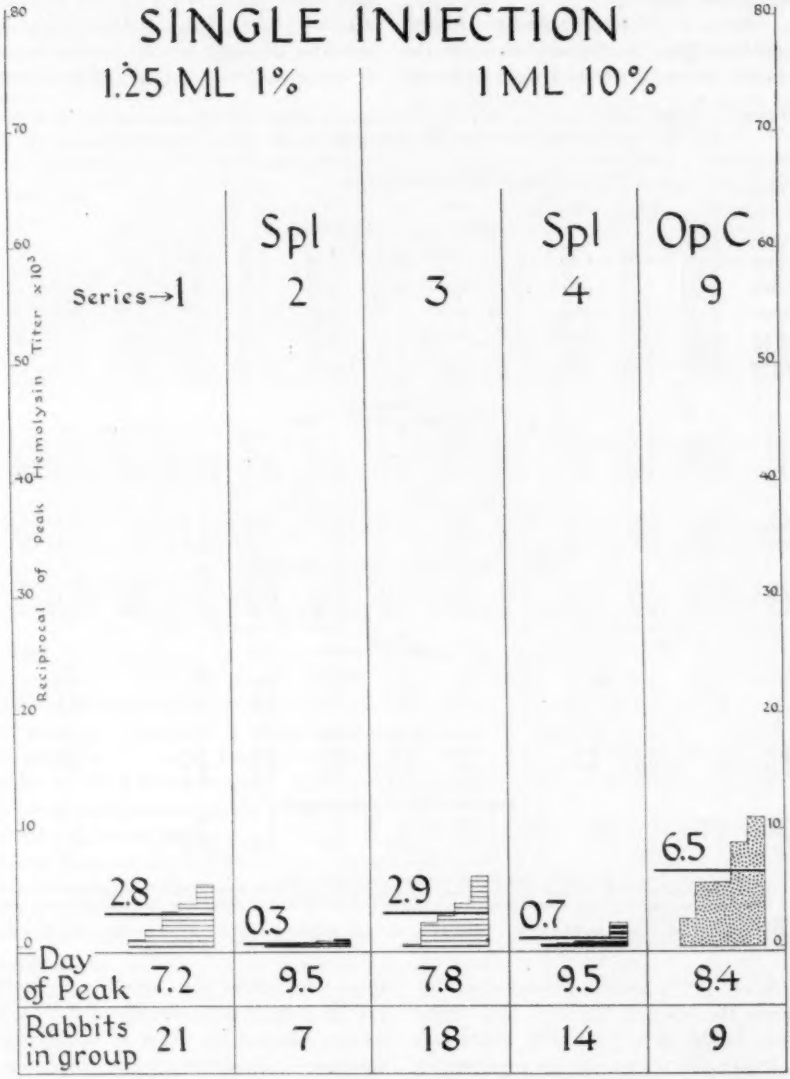
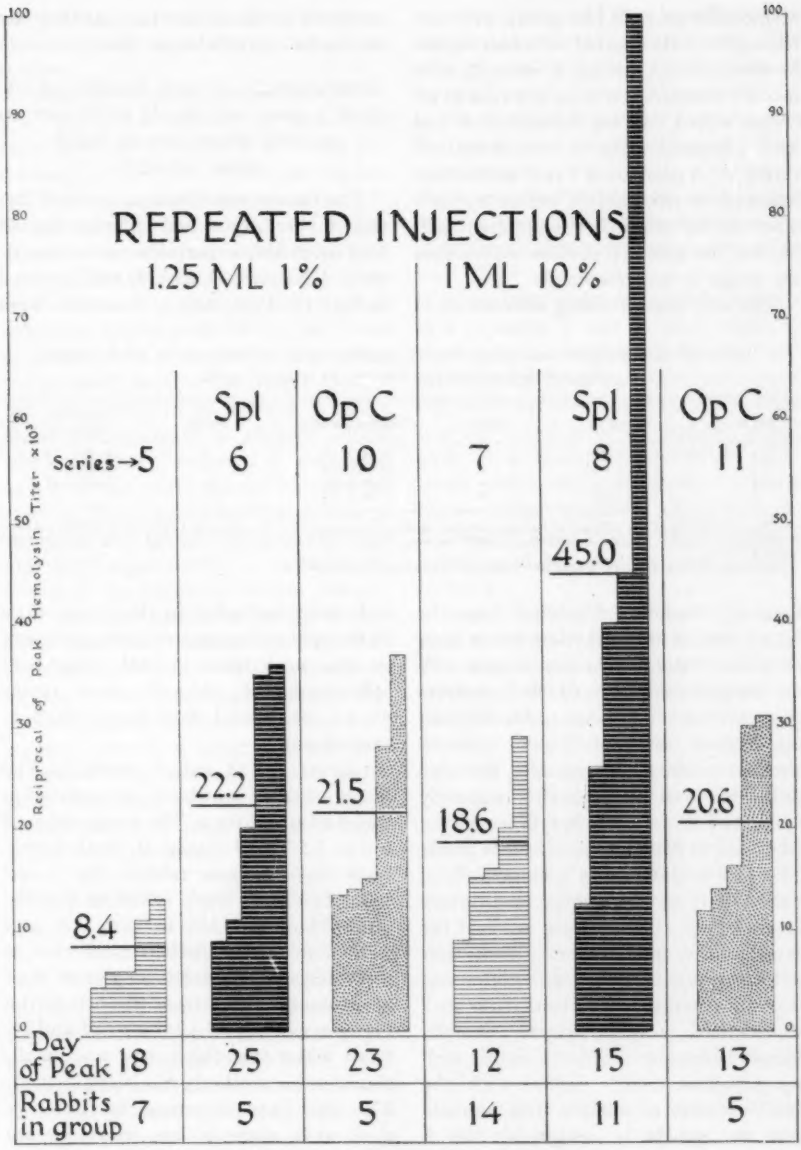


FIG. 1.—Peak hemolysin titers in 11 series of rabbits which differed as to whether they were intact, splenectomized or operative controls and as to whether they received single or multiple injections of 1.25 ml 1% or 1 ml 10% sheep red blood cells per kg. In addition, mean peak titer, mean day of peak titer and the number of rabbits in each series are shown. The 5 rabbits in most series are representative of larger numbers, but series 6, 10 and 11 consisted of only 5 rabbits each. The titers and times



are from fitted curves. Op C=operative controls, i.e., a testis, kidney or the appendix removed; Spl=splenectomized.

Note that splenectomy delayed the peak titer in all series but lowered it after a single antigen injection and raised it after repeated antigen injections.

Hemolysin titers in this group were low throughout the period of observation. Peaks (table 1A and fig. 1, series 2) were usually reached at a constant rate of increase with k varying between 0.06 and 1.67. The mean value for k was 0.8 ± 0.18 (table 2). A plateau of 1 to 3 weeks then followed in two rabbits before the primary decline started. The apparent half life for the primary decline was within the range in intact animals.

The two most striking differences in these splenectomized rabbits from the intact ones in the preceding series were (1) a lower titer for the first month with the highest difference (2330 hemolysin units/ml) on the 8th day (table 1B) and the highest ratio of intact: splenectomized rabbits (34) on the 6th day (table 1C) and (2) peak titer was only a ninth of that in intact rabbits with a difference of 2500 hemolytic units (table 1B). Other differences included (1) a lower value for k during the primary antibody rise, (2) a longer interval between initial injection and peak titer and (3) the occurrence of a plateau of long duration after the peak titer in 2 rabbits with a later primary decline. These results are in general accord with our previous results (1950) with the small amount of antigen and indicate that the spleen is responsible for a major portion of the initial antibody rise after one injection of a small number of sheep cells. As we shall stress later, nonsplenic sites eventually form as much antibody as the spleen because they are active for a much longer time.

Experiment 2.—A single injection of 1 ml of 10% sheep red cells/kg rabbit into intact and splenectomized rabbits (series 3 and 4)

The same relationships existed between the intact and splenectomized rabbits in this experiment as in experiment 1 (cf series 1 with 2, and 3 with 4 in fig. 1). Peak titers, however, were only 4 times higher in the intact than in the splenectomized animals, and peak or near peak titers in both intact and splenectomized animals were maintained for several days longer than in experiment 1.

Series 3 (18 intact rabbits).—The initial rate of antibody accumulation was 0.67 to 2.7 for k. The mean value of k was 1.57 ± 0.9 (table 2). Peak hemolysin titers in these rabbits (fig. 1 and table 1) varied from 1:200 to 1:6000, occurred on the 5th to 12th day and were more often reached after two or three successively decreasing rates than after one (cf titers from the 6th to the 12th days in table 1A for series 1 and 3). Even when this third rate was small, considerable antibody was being formed. The titer then decreased for about a week at a more or less constant rate with an apparent half life of 5 to 12 days (mean of 7.95 ± 0.4 days). This less rapid decline as compared to animals receiving smaller amounts of antigen

TABLE 2.—*Mean values* and standard errors for the constant of increment (k) for the initial phase of the antibody rise from the 11 series of rabbits.*

Series	Intact	Series	Splenectomized	Series	Operative controls
1	1.40 ± .05†	2	0.80 ± .18	9	2.39 ± .14
3	1.57 ± .09†	4	0.88 ± .15	10	1.86 ± .11
5	1.47 ± .11	6	1.16 ± .11	11	1.37 ± .15
7	1.58 ± .16	8	1.30 ± .14		

* The significance of the differences between various series are indicated by the following values for $P \leq 0.001$ between series 1 and 2, 3 and 4, 9 and 3, and 10 and 1; ≤ 0.05 between 2 and 8 and 4 and 8; > 0.1 for series 1 and 3, 2 and 4, 7 and 3, and 7 and 11.

† For these values, 35 extra rabbits were added to series 1 and 14 to series 3.

we (1950) ascribed to the continued elaboration of antibody by nonsplenic sources.

Series 4 (14 splenectomized rabbits).—The greatest difference in hemolysin titer between the intact and splenectomized rabbits occurred on the 8th day (1850 units) and the largest ratio (15) on the 6th day (table 1B and C). The initial rate of increase varied from 0.16 to 1.76. The mean was 0.88 ± 0.15. Two of the 14 splenectomized rabbits had definitely higher peak titers than those in series 2. The apparent half life during the primary decline was longer than in the intact rabbits of series 3 (10.6 ± 2 days), and plateaus or periods during which antibody increased or decreased at a slow rate preceded the primary decline in 11 of the 14 rabbits. Although the peak titer of the intact rabbits did not differ significantly in experiments 1 and 2, the following results indicate that the larger amount of antigen stimulated nonsplenic sources to form both absolutely and relatively more antibody in the splenectomized rabbits: The splenectomized rabbits in series 4, as compared to the splenectomized rabbits in series 2, occasionally had a higher peak titer, more commonly a plateau or virtual plateau at peak titer, and a slower antibody decline.

Experiment 3.—Ten injections of 1.25 ml of 1% sheep red cell/kg rabbit into intact and splenectomized rabbits (series 5 and 6)

This experiment involved multiple injections of antigen of the intermediate amount of red cells instead of a single injection as in experiment 1. The second injection followed the first one after one week: the last 9 injections were given three times a week. A week was allowed to elapse between the first two injections so that the initial rise would be largely the result of a single antigen injection and would then be followed by repeated antigenic stimulations. The schedule is shown at the bottom of figures 2 and 3. The rabbits weighed 2.1 to 2.8 kg when first injected and 2.5 to 3.4 kg at the last injection. As mentioned previously, statements of time in these repeatedly injected rabbits will refer to intervals after the first injection of antigen unless expressly stated otherwise.

In contrast to the singly injected rabbits in series 1 and 2, peak titers in repeatedly injected splenectomized rabbits were 2.6 times higher than in repeatedly injected intact ones (cf series 5 and 6 in fig. 1 and table 1A and B). Moreover, as a result of the multiple injections, mean peak titers were 3 times higher in intact rabbits (cf series 1 and 5 in fig. 1) and were 74 times higher in splenectomized ones (cf series 2 and 6 in fig. 1).

Series 5 (7 intact rabbits).—Hemolysin titers for the first week were within the range of those in series 1 (table 1A) but increased after further antigenic stimulation to peaks of 1:4200 to 1:14,000 between the 13th and 28th days. There were two or more changes in rate of antibody rise (fig. 2) before the primary (the most rapid) decline set in. The peak was more or less maintained during the period of antigen stimulation in 5 of the 7 rabbits and then declined immediately or within a week (fig. 2B and C). One rabbit died shortly after peak titer. In the 7th rabbit (117, fig. 2A), the titer rose to 1:13,000 by the 13th day and decreased slowly (apparent half life of 24.8 days) in spite of continued antigenic stimulation. This slow decline was maintained for 13 days after injections had been stopped. Then a more rapid phase set in (apparent half life of 8.2 days). The apparent half life of the primary decline in 4 of the rabbits varied between 7.5 to 9.6 days and in two

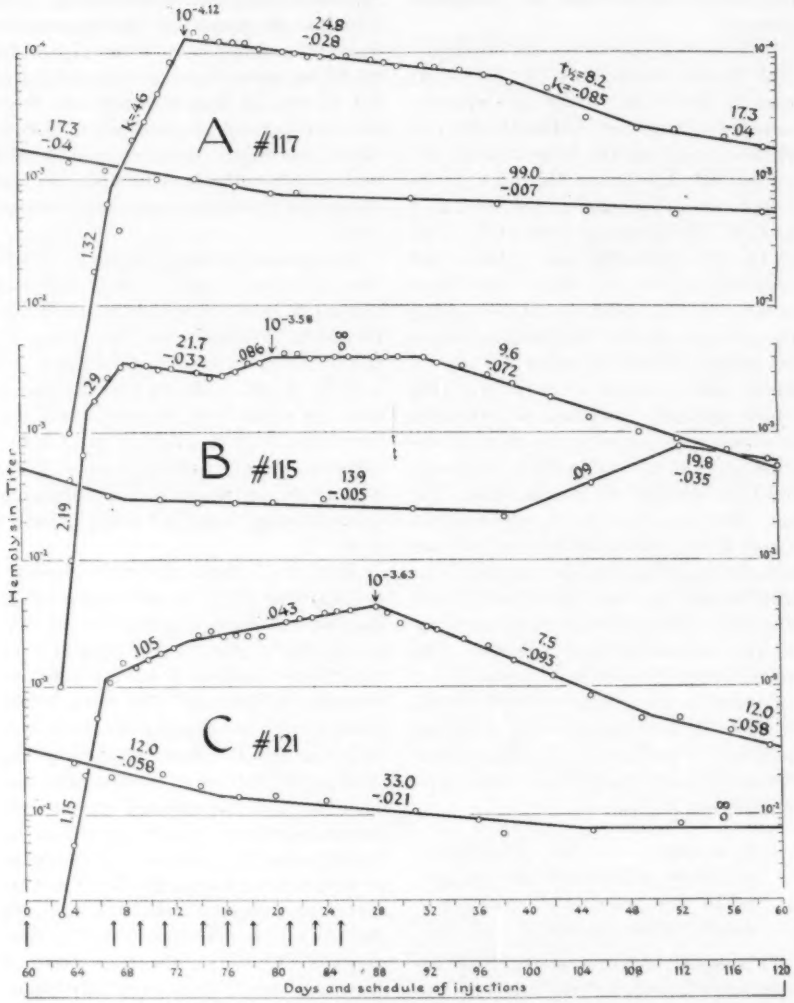


FIG. 2A, B and C.—Changes in hemolysin titer for 120 days in intact rabbits 117, 115 and 121 of series 5.

Arrows at the bottom of the figure indicate the time of injecting 1.25 ml 1% sheep red cells intravenously per kg rabbit. Peak titers are given from the fitted curves. The rate of hemolysin accumulation for the initial rise is indicated by values of k (constant of increment), and successive rates of antibody decline are indicated by the apparent half life in days (upper figure) and by the negative value of k (lower figure).

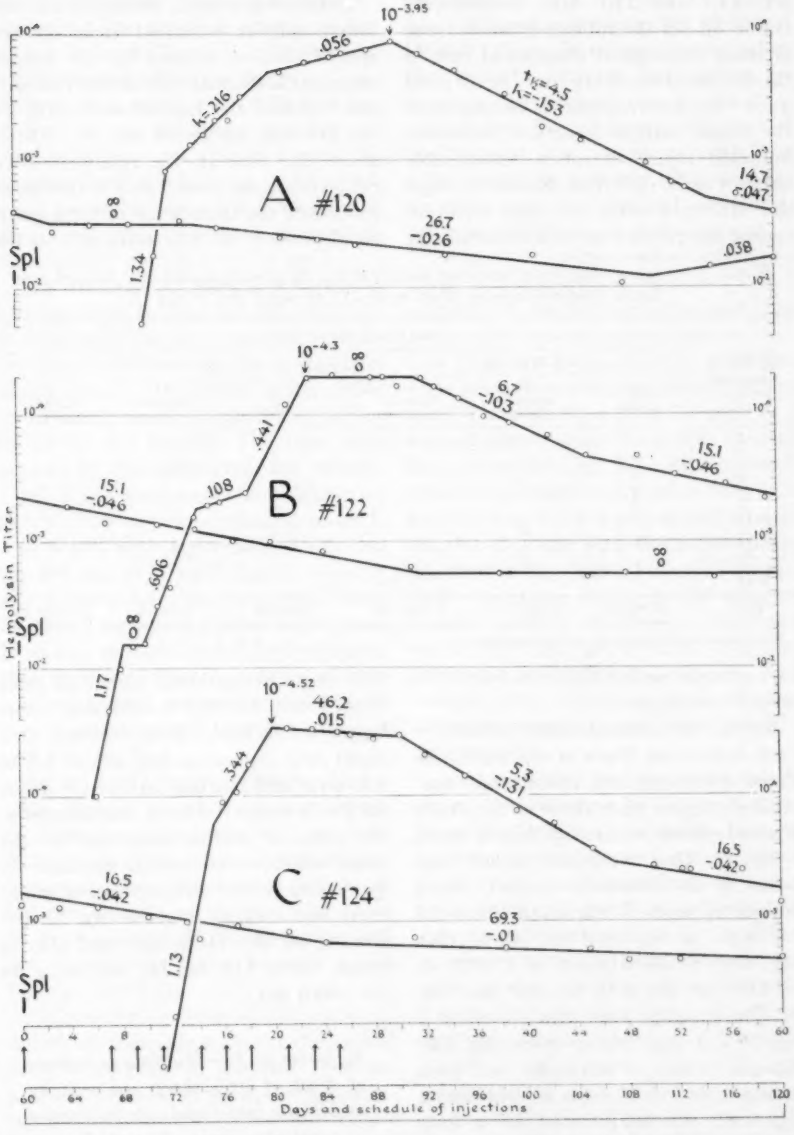


FIG. 3A, B and C.—Changes in hemolysin titer for 120 days in splenectomized rabbits 120, 122 and 124 of series 6. These rabbits were injected as were those in figure 2. See figure 2 for other explanations.

Hemolysin, after a longer induction period than in the intact rabbits in figure 2, reached higher peaks.

was 14.7 and 23.1 days, respectively (table 3). All the rabbits exhibited one or more subsequent changes of rate in the decline of antibody titer (fig. 2) until a low rate was reached. As compared to the single antigen injection, therefore, multiple injections of a comparable amount of red cells from the 2nd through the 4th week after the first injection caused the production of more antibody over a longer period (cf series 1 and 5 in table 1 and figure 1).

In this experiment, hemolysins in the intact rabbits exceeded those in the splenectomized rabbits by the largest amount (3240 units/ml) on the 12th day and reached the highest ratio (76) on the 8th day (table 1B and C). Thereafter, the titer in the splenectomized rabbits rose, surpassed that in the intact rabbits on the 18th day, reached a greatest difference (13,900 units/ml) on the 24th day and continued higher for some time. From the 30th to 35th day, titers began to decline. Three declined at a rapid rate (apparent half life of 4.5 to 6.7 days) and two less rapidly (11 days) for 2 to 3 weeks (table 3). Subsequently, the rates of decline proceeded in the usual manner—at several successively decreasing rates—although occasionally there was a slight increase (fig. 3A beginning on the 110th day and also in intact rabbit 115, fig. 2B, beginning on the 100th day).

TABLE 3.—*Apparent half life of hemolysin in days during the primary decline after the peak titer in the repeatedly injected rabbits of series 5 through 8 and 10 and 11.*

Apparent half life of hemolysin in days	Red blood cells per injection					
	1.25 ml 1%			1 ml 10%		
	5 Intact	10 Operative controls	6 Splenectomized	7 Intact	11 Operative controls	8 Splenectomized
5			2			
6				2	1	
7			1	2	1	1
8	2	1			1	
9	1			3	1	
10	1			1	1	
11			2	1		3
12		1		3		
13		1				3
14		1				
15	1	1				1
>15	1*					
Mean	12±2.44	12.6±1.24	7.8±1.35	9±0.65	8±0.70	11.7±0.84

* In this rabbit, the apparent half life was 23 days.

Series 6 (5 splenectomized rabbits).—Peak hemolysin titers in repeatedly injected splenectomized rabbits were surprisingly higher than those in the singly injected splenectomized rabbits of series 2 (fig. 1). They were also higher than those in the similarly treated intact rabbits of series 5. Although titers did not begin to rise until the 7th to 12th day, they reached titers of 1:8900 to 1:34,000 on the 20th to 33rd day (fig. 3). These peaks were attained after 2 (fig. 3C), 3 (fig. 3A) or more (fig. 3B) changes in rate of antibody rise, each lasting from 3 to 10 days. In addition, a high titer was maintained for a long time in all rabbits. A slow increase (fig. 3A), plateau (fig. 3B) or slow decline (fig. 3C) lasted for 1 to 2 weeks before the primary rapid decline set in.

Experiment 4.—Multiple injections of 1 ml of 10% sheep red cells/kg rabbit into intact and splenectomized rabbits (series 7 and 8)

This experiment was carried out with two batches of rabbits. In the first batch (rabbits 164 through 172), 34 in-

jections were given during 10.5 weeks according to the schedule shown at the bottom of figures 4 and 5. In the second batch (rabbits 179 through 194), 17 injections were given during 34 days according to the following schedule: 3 daily injections and 3 times a week for 5 weeks. Each injection was equal to the single one in experiment 2. Antigen was given frequently at first in contrast to the schedule used in experiment 3.

The following differences were found between these rabbits and the previously described ones (fig. 1 and table 1). In the intact rabbits, hemolysins appeared sooner, reached uniformly higher peaks sooner and remained appreciably higher for about two months. The same may be said for the splenectomized rabbits. Thus, titers were appreciably higher on the 4th day in series 7 than in series 1, 3 or 5 and were appreciably higher on the 8th day in series 8 than in series 2, 4 or 6 (table 1A). Similarly, peak titers in series 7 were over 6 times higher than in series 1 or 3 and were 2.2 times higher than in series 5 (fig. 1). For the splenectomized groups, the ratios were 150, 64 and 2, respectively (cf series 8 with 2, 4 and 6 in fig. 1).

Series 7 (14 intact rabbits).—Variations during hemolysin accumulation in these intact rabbits may be seen in figure 4. The primary declines unexpectedly started in 6 to 15 days in all but two rabbits as compared to 13 to 28 days in series 5. The apparent half life of antibody varied between 6 and 12 days (table 3).

Fluctuations in titer sometimes made it difficult to decide upon the theoretical rates of increase or decrease. For example, the series of points in figure 4C between the 7th and 42nd day might have been connected by lines giving two changes in rate with successive half lives of 22.3 and 10.5 days or even by lines giving 3 changes in rate with successive half lives of 22.3, 5.9 and 12.0 days. Also, the points between the 42nd and 56th day might have been connected to give a slight increase and decline instead of the plateau shown. We considered such fluctuations a reflection of the frequent injection of red cells. The main finding is, however, that hemolysin titers definitely declined while antigen was still being regularly injected.

Series 8 (11 splenectomized rabbits).—Data in table 1A and figures 1 and 5 corroborate the findings in series 6 that repeatedly injected splenectomized rabbits begin to form antibody later but form more than similarly injected intact ones. Titers declined after the peak at a comparatively slow rate (table 3) in all but one rabbit (fig. 5C). After a maximal excess of 6560 units/ml in the intact rabbits over the splenectomized ones on the 7th day and with the highest ratio on the 5th day (table 1B and C), antibody then rose higher in the splenectomizd rabbits, attained a maximal excess of 25,800 units on the 20th day and remained higher for 2 months. At 4 months titers were about the same in series 5 through 8 and were generally below 1:1000.

In considering the statistical significance of differences in the peak titers between the intact and splenectomized rabbits of experiments 3 and 4, actual peak titers and logarithms of actual peak titers were used. Logarithmic values give a relative value of the differences and permitted combining the two experiments. Actual peak titers for the repeatedly injected intact and splenectomized groups showed differences of $13{,}800 \pm 5940$ units ($P = 0.05$) and $26{,}400 \pm 9780$ units ($P = 0.02$), respectively (table 1B). When the two groups were combined, the logarithm of titer for the splenectomized group (4.47) was higher than for the intact one (4.11) by 0.36 ± 0.08 with $P = 0.001$.

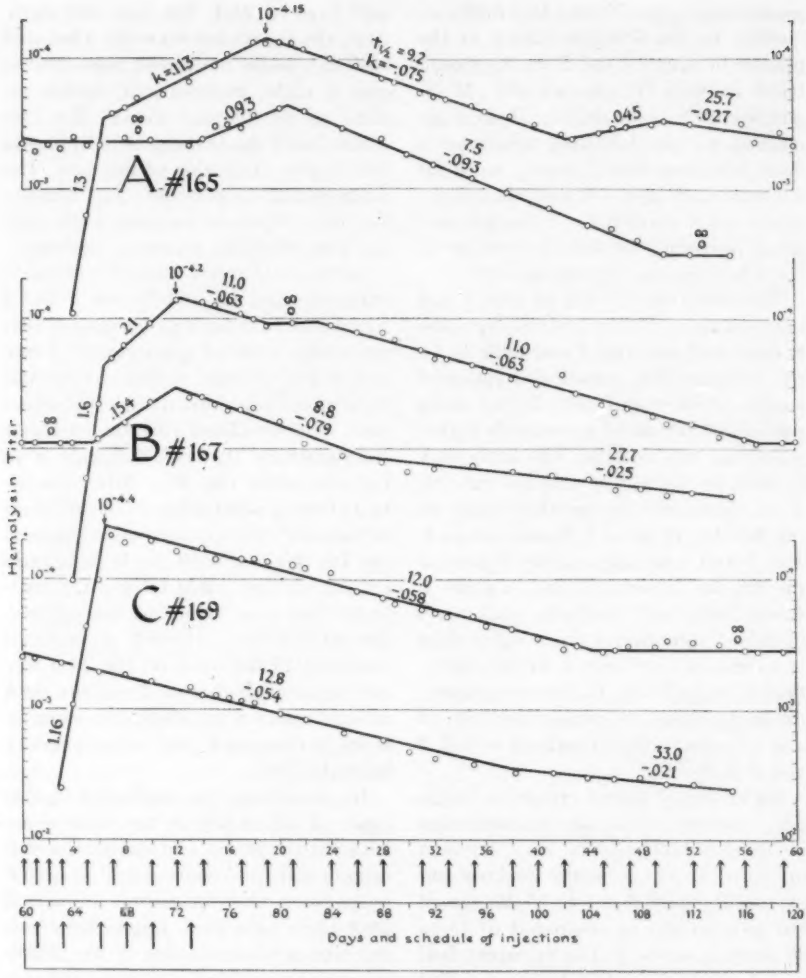


FIG. 4A, B and C.—Changes in hemolysin titer for 116 days in intact rabbits 165, 167 and 169 of series 7. Arrows at the bottom of the figure indicate the time of injecting 1 ml 10% sheep red cells intravenously per kg rabbit. See figure 2 for other explanations.

Hemolysin titer began to decline in spite of continued antigenic stimulation.

Experiment 5.—Operative controls (series 9, 10 and 11)

In our earlier work (1950), some of the highest titers reached after single antigen injections occurred in animals after the removal of the appendix, a kidney or a testis. At the time, it did not seem important to pursue this work because such stimulation, if present, would indicate that the depressing effect of splenectomy found in rabbits

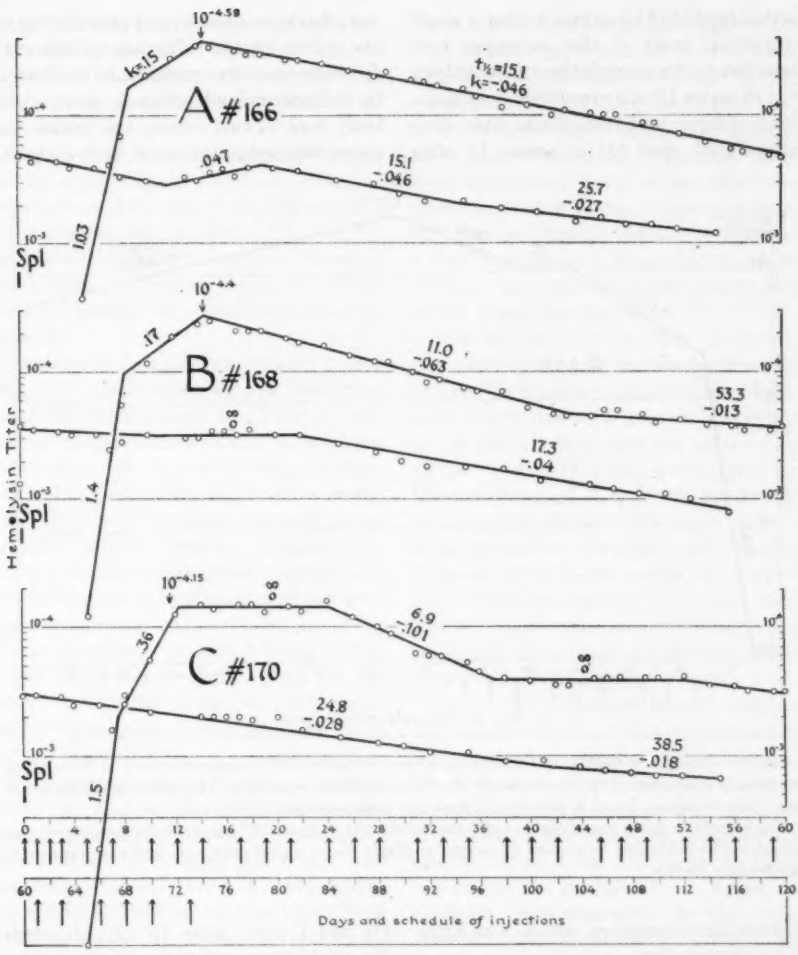


FIG. 5A, B and C.—Changes in hemolysin titer for 116 days in splenectomized rabbits 166, 168 and 170 of series 8. These rabbits were injected as were those in figure 4. See figure 2 for other explanations. Splenectomy enhanced the production of hemolysin as in series 6. As a result of more intense antigenic stimulation than in series 6, antibody appeared earlier, rose at similar rates for longer periods to higher peaks, and declined from the peaks sooner although at a slower rate in two (166 and 168) of the three rabbits.

receiving a single injection of antigen was even more significant than the results indicated. With the reversal of the effect of splenectomy in singly and repeatedly injected rabbits, however, the question of possible stimulation by the operation became of great importance.

At present, it is impossible to evaluate the effects of the operation exactly. Results from the following three series

indicate that (1) in series 9 after a single injection, most of the increased titer was due to the stimulation of the spleen, (2) in series 10 after multiple injections, both splenic and nonsplenic sites were stimulated, and (3) in series 11 after intense immunization, there was little or no stimulation.

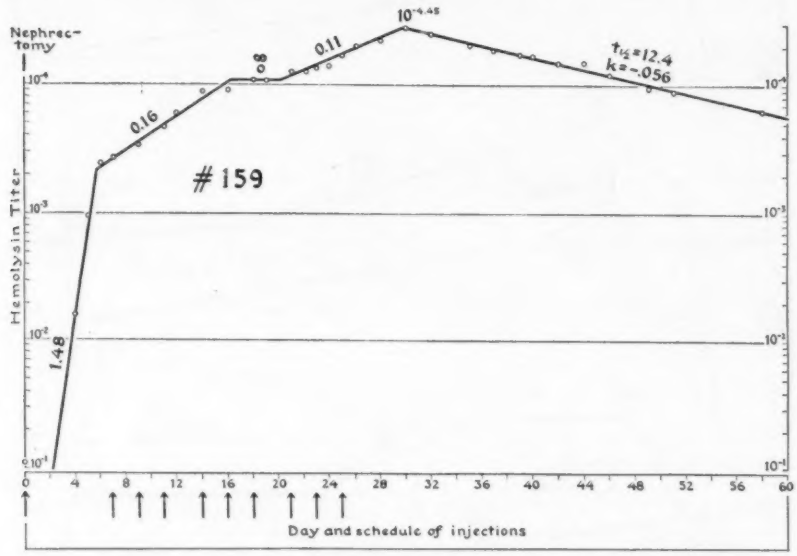


FIG. 6.—Changes in hemolysin titer during 60 days in rabbit 159, an operative control, from which one kidney was removed two hours before the initial injection of antigen. This rabbit was injected as were those in figures 2 and 3. See figure 2 for other explanations.

The hemolysin curve resembles that of intact rabbit 121 in figure 2C, but antibody production was stimulated as evidenced by a more prolonged antibody rise, a higher peak titer and a slower rate of the primary decline.

Series 9 (9 operative controls injected singly as in experiment 2).—As seen in table 1A, B and C, the mean peak titer in the operative controls (3 each from which the appendix, a kidney or a testis was removed) was over twice that of the 18 intact animals in series 3 receiving the same amount of antigen. The difference amounted to 3630 ± 1110 hemolytic units/ml with $P = 0.005$. This excess of hemolysin must, to a large extent, have resulted from a stimulation of the spleen for the following reasons: (1) In these operative controls as compared to splenectomized series 4, more antibody was formed during the initial rise when the spleen is most active (table 1B and C and table 2). (2) Although the peak titer and rates of the initial rise and primary decline were higher in the operative controls than in intact rabbits, the shape of the antibody curve for the first month (see fig. 4 of our 1950 paper) is what would be expected from increased splenic activity. (3) The apparent half life of the primary decline (6.5 ± 0.3) was significantly shorter than in intact animals (7.95 ± 0.4) as pointed out in our earlier paper. This would be expected if nonsplenic sources continued

to be active in both groups while a larger proportion of splenic antibody in the serum of the operative controls was undergoing metabolic decay without replacement.

Series 10 (5 operative controls injected as in experiment 3).—Three gonadectomized rabbits and 2 nephrectomized rabbits were injected with 1.25 ml of 1% sheep red cells in a manner identical with that used in experiment 3. Two intact rabbits were injected simultaneously.

As shown in table 1A, B and C and figure 1, peak titers of the operative controls were equal to those in the splenectomized animals and were higher than those of the intact ones. A considerable part of the high titers in the operative controls, however, was due to greater splenic activity, whereas the high titers in the splenectomized animals were due to the stimulation of nonsplenic sources (cf fig. 6 with 2 and 3). This conclusion is based upon differences in the mean value for k of the primary rise (table 2) and in daily mean titers of series 5, 6 and 10 (table 1B). Thus, assuming that splenectomy simply prevents splenic activity, splenic antibody can be approximated by subtracting the daily mean titers of splenectomized series 6 from the intact and operative series. This is particularly true during the first 10 days when little antibody was found in the splenectomized series. For example, the spleen formed about 3040 units/ml in intact animals by the 10th day and about 4530 units or 50% more, in the operative controls. It also formed about 4090 units more in the operative controls than in intact animals of the singly injected series. It is risky to continue such an analysis after nonsplenic sources become highly active and after the spleen probably stops forming antibody. Nevertheless, at the time of the greatest difference (6340 units) between series 5 and 10 on the 16th day, the spleen seemed to have formed twice as much antibody in the operative controls as in the intact rabbits. By this time, nonsplenic sources were also stimulated. Whether they were stimulated more or less than the spleen is difficult to decide, because they only reached maximal activity later at a time when it was impossible to know whether the spleen was still stimulated in the operative controls.

One further fact should be noted, especially in view of the results in series 11. The high titers in series 10 may have been partly due to a greater reactivity of the rabbits than was encountered in series 5 and 6. This idea is suggested by the fact that peak titers in the two intact rabbits immunized at the same time as series 10 were in the high range reached in series 5. Such differences are part of the baffling variability we have constantly found in the amount of antibody produced in different rabbits under standardized conditions.

Series 11 (5 operative controls injected as in experiment 4).—The previous work indicated that results were the same when the appendix, a testis or a kidney was removed. In view of the fact that the testis weighs about the same as the spleen, we removed one testis via laparotomy in the rabbits of this series. In addition, we immunized at the same time the second batch of rabbits described in experiment 4. Six were splenectomized and 10 were left intact. Peak titers in the intact and splenectomized animals each formed a continuous series with the earlier tested rabbits in series 7 and 8, respectively, and indicated that the reactivity of the two batches was essentially similar.

Examination of the data in table 1A and B indicates the following: The mean peak titer of the intact and the operative controls did not differ significantly

(2000 ± 4420 units/ml), whereas the mean peak titer of splenectomized series 8 was significantly higher than either intact series 7 ($26,400 \pm 9780$ units/ml with P about 0.02) or the operative control series 11 ($24,400 \pm 10,390$ units/ml with P about 0.04). Furthermore, a comparison of daily mean titers and of the ratios in table 1A, B and C, as described in the previous section, indicates that the spleen in the operative controls was not stimulated and that nonsplenic sites were stimulated to a very minor extent. Thus, contrary to the findings in series 10, this series indicates that most, if not all, of the effects of splenectomy were due to removal of the spleen. These results may more nearly reflect the true situation. Until further work is done, however, weight should be given to both series. This procedure takes into account the undoubted stimulation of the spleen in the singly injected rabbits of series 9.

In order to get a relative value of the effects of the operation and splenectomy, we combined series 5 and 7 (intact), 6 and 8 (splenectomized), and 10 and 11 (operative controls) and have compared logarithms of the peak titers. The results indicate the following: The largest and statistically most significant difference was obtained between the splenectomized and intact animals. The difference was 0.36 ± 0.08 with $P = 0.001$. A smaller and statistically less significant difference occurred in the operative controls and intact animals. This difference was 0.17 ± 0.07 with $P = 0.02$. About the same difference (0.19 ± 0.08) was observed between the splenectomized and operative controls with $P = 0.03$.

DISCUSSION

These results with single (see also our work, 1950) and multiple injections will be considered with especial reference to: (1) the nature of the curve describing the rise and fall of antibody, (2) the role of the spleen and the hypothetical separation of splenic from nonsplenic antibody, (3) the increasing relative importance of nonsplenic sites in antibody formation as antigenic stimulation is increased, (4) the reversal of the effects of splenectomy as antigenic stimulation is increased, and (5) a tentative working hypothesis to integrate the experimental findings obtained so far. To facilitate comparisons of the different series and to evaluate the activity of the spleen and the possible stimulating effect of the operation, the material in table 1 will be used as a basis for much of the following discussion without extensive specific references to it.

A comparison of figures of singly injected animals in our earlier paper (1950) with figures 2 through 5 of repeatedly injected intact and splenectomized rabbits in the present paper indicates that the data from the singly injected animals fit the theoretical lines of log titer plotted against time much better than those from the repeatedly injected animals. We believe this difference is real and cannot be explained by differences in experimental technique. The same workers performed the tests for the series in figures 2 and 3, which showed the greatest scatter, as for the tests in our first paper. Some of the aberrant points may be accounted for by the interference of transient negative phases because of an immediately preceding injection and upthrusts of antibody production because of injections 4 to 7 days previously. To minimize variations, blood samples were usually drawn early in the day and injections were made around noon. Titers in the present work, however, fall on the curves in a surprisingly regular

fashion considering the fact that the rabbits were receiving repeated antigenic stimulation for almost a month in figures 2 and 3 and for over half of the time covered by the graphs in figures 4 and 5. Put another way—the remarkable finding is not the scatter of the data from the repeatedly injected animals, but the almost perfect fit of those from the singly injected ones.

That repeated antigen injections were holding the titer down is suggested by the rises in titer at the end of the immunization period in rabbits 165 and 166 (fig. 4A and 5A) from 72 through 80 days but is not borne out by the mass of data, as most animals do not show such rises. In addition, the same type of rise occurred in rabbit 165 (fig. 4A) from 42 through 49 days while antigen was being injected 3 times a week and in rabbits 115 (fig. 2B) and 120 (fig. 3A) 74 to 87 and 85 to 95 days after the last antigen injection, respectively. It also occasionally occurred after a single injection of antigen (cf rabbit 4 in fig. 1 of our paper on the effects of X-irradiation, 1950b).

In our previous study with single injections of antigen (1950), we assumed a simple additive relationship between splenic and nonsplenic antibody sources. The average peak titer in splenectomized animals with the large amount of antigen was about 20% that in intact animals. Furthermore, since serum antibody in intact animals disappeared after peak titer at a logarithmic rate indicating a half life of the order of magnitude accepted for serum globulins and since splenectomy at about peak titer did not increase the rate of disappearance, we assumed that the spleen abruptly stops or almost stops forming antibody. The subsequent plateau or slow decline, which was roughly equivalent to that encountered in splenectomized animals, we believed represents the low grade but continuing activity of nonsplenic antibody sources.

The same general type of analysis can be made by comparing serum titers in the repeatedly injected intact and splenectomized animals in the present paper although complicated by the fact that nonsplenic sites were more active in the splenectomized rabbits, as indicated by higher titers, than in the intact rabbits. Serum titers represent the total antibody produced minus that lost by metabolic decay. The total antibody formed during the initial antibody rise can be expressed by the following equation:

$$S = A + B \tag{2}$$

where S represents the total antibody formed, A the observed antibody accumulating in the serum after immunization ($A_t - A_0$), and B the antibody destroyed.

If k_1 and k_2 are rate constants for A and B, respectively, the change in concentration during the rise in antibody is observed to be

$$A_t = A_0 e^{k_1 t} \text{ (from equation 1, page 143)} \tag{3}$$

The quantity of antibody destroyed is proportional to the antibody present. Therefore,

$$\frac{dB}{dt} = k_2 A \tag{4}$$

$$\frac{dB}{dt} = k_2 A_0 e^{k_1 t} \text{ (by substitution from 3)} \tag{5}$$

$$B = A_0 \frac{k_2}{k_1} (e^{k_1 t} - 1) \tag{6}$$

The total antibody synthesized between time 0 and time t will be

$$S_t = (A_t - A_0) + B_t \tag{7}$$

$$= A_0 (e^{k_1 t} - 1) + A_0 \frac{k_2}{k_1} (e^{k_1 t} - 1) \tag{8}$$

$$= A_0 \frac{k_1 + k_2}{k_1} (e^{k_1 t} - 1) \tag{9}$$

In most cases, equation 9 can be simplified to

$$S_t = \frac{k_1+k_2}{k_1} A_0(e^{k_1 t}) \tag{10}$$

$$= \frac{k_1+k_2}{k_1} A_t \tag{11}$$

If we assume that the preimmunization titer (A_0) and metabolic decay are similar in different animals, the amount of antibody undergoing decay would increase relatively as k_1 (the rate of observed antibody rise) decreased. Thus, peak titers given in table 1 would range from 5 to 16% more if $k_2 = 0.126$ and $t_{\frac{1}{2}} = 5.5$ days in all animals. In series 3 and 4, the percentage increase would be 8 and 14%, respectively. Thus, ratios between the observed serum (A_t) titers are approximately correct during the initial rise.

The differentiation of splenic and nonsplenic sources of antibody formation in experiment 3 is interesting because antibody curves for the first week and probably for one or two more days can conservatively be considered to be influenced only by the first antigen injection. In fact, the similarity between the differences in titer through the 8th day for the intact and for the splenectomized rabbits in experiments 1 and 3 is striking. The similarity then vanishes as the titer in the splenectomized rabbits of experiment 3 reached and exceeded the titers in the intact rabbits. Antibody curves in the intact animals can be reasonably explained by a cessation of splenic activity on about the 12th day, when the greatest difference (3240 units/ml) occurred, and an undoubted continuing activity of nonsplenic sites (fig. 3) which then accounts for a progressively increasing proportion of serum antibody. The cessation of splenic activity and the subsequent disappearance of splenic antibody with a half life of 5.5 days superimposed on the continuous activity of nonsplenic sites would give the plateaus or slowly changing antibody levels observed during the first month (fig. 2).

Little is gained by trying to interpret the ratios in experiment 3 after hemolysins have begun to decrease because varying amounts of residual splenic antibody may be present after the partial or complete cessation of splenic activity. On the other hand, it is instructive to note the character of the antibody rise in the intact animals of this experiment. The first phase of the initial rise was statistically the same as that in singly injected intact animals both in regard to time and size of k (table 2), but the seconday phase was sometimes prolonged. As illustrated in rabbit 117 (fig. 2A), the second phase of the initial rise lasted almost 6 days with $k = 0.46$. All of this secondary phase was probably largely due to the activity of the spleen, since the rate of rise continued at an unchanged rate during a period when antibody accumulation had not gained a high momentum in splenectomized animals (fig. 3). A third phase of the rise, however, as in rabbit 121 from the 13th to the 28th day, was probably largely due to nonsplenic sources because of the high activity in splenectomized animals during this period. A curve such as found in rabbit 115 (fig. 2B) could result from the following: the cessation of splenic activity on the 8th day, as found after a single antigen injection, little increased splenic activity as a result of the multiple injections during antibody decline from the 8th to the 16th day and the stimulation of nonsplenic sources on about the 18th day.

In contrast to the repeatedly injected animals of experiment 3, in which the initial rise through 8 days was only influenced by the initial antigen injection, the rabbits in experiment 4 received 3

or 4 daily injections of a larger amount of antigen (1 ml of 10% cells) initially and a longer course of injections. As a result of this greatly increased stimulation, serum titers were higher. The rates of increase were similar, but continued for a longer time. If splenic function is additive, as we have assumed, repeated antigen injections during the first few days stimulated the spleen to greater activity than a single smaller antigen injection as evidenced by the following: (1) The first phase of antibody rise, with the highest value of k, was terminated on the 6th day (fig. 4), and by the 7th day the average titer in the intact animals (1:9530) exceeded that in the splenectomized animals (1:2970) by the greatest difference (6560 hemolytic units/ml). (2) The amount thus ascribable to the spleen was about twice the highest amount ascribable to the spleen in experiment 3 (during the influence of the first antigen injection) on the 12th day (3240 units) and about 2.8 times that in experiment 1 on the 8th day (2330 units).

In addition to a higher splenic activity, the frequent initial antigenic stimulation in experiment 4 increased the relative importance of nonsplenic sources for a month or two, as shown by higher titers in the splenectomized rabbits and a greater speed of reaching titers equal to those in intact rabbits. Thus, the highest average titer was 38,300 units on the 19th day in series 8 as compared to 18,380 units on the 23rd day in series 6, and a ratio of about 1 was reached around the 10th day in experiment 4 and not until the 16th day in experiment 3.

The assumption that the spleen largely stops forming antibody is in line with the work in our previous paper (1950) with singly injected animals and is specifically based on the following facts: Serum antibody after peak titers in repeatedly injected intact animals (table 3, series 7) declines at a rate similar to the primary decline ($t_{\frac{1}{2}} = 7.95 \pm 0.4$ days) in singly injected animals (series 3) in which the evidence is good that the decline is due to the decay of splenic antibody without appreciable replacement. Nonsplenic antibody continues to be formed as indicated by the later attainment of peak titer and the slower decline from peak titer in the splenectomized repeatedly injected animals of series 8. The rate of the primary decline in the repeatedly injected and singly injected splenectomized series is similar (cf table 3 with table 10 of our earlier paper, 1950) but involves the production of more antibody in the former because of the higher titers.

Splenic antibody has been separated from nonsplenic antibody in the intact rabbits of experiment 4 according to the following propositions: (1) It is obvious that a separation cannot be made by a simple subtraction of titers in splenectomized animals from those in intact animals because the splenectomized animals eventually formed more antibody and, hence, nonsplenic sources must be more active in them than in intact animals. To obtain splenic antibody, we have subtracted the titers of splenectomized animals in series 8 from intact animals in series 7 only through the 7th day, which is the day of the greatest difference. These 7 days represent a period of very little nonsplenic activity (see series 8). (2) From the 7th day on, it is assumed that the spleen stops forming antibody and that splenic antibody disappears with a half life of 5.5 days. The assumed half life of 5.5 days is the best approximation we could obtain in the presence of a low activity of nonsplenic sources (see our paper, 1950). (3) From the 7th day on, nonsplenic antibody each day is assumed to be the difference between the actual mean titer

in the intact rabbits and the assumed residual amount of splenic antibody.

The hypothetical separation of antibody formed in the spleen from that formed elsewhere in the intact rabbits of experiment 4 is shown graphically in figure 7. The curve of nonsplenic antibody is a reasonable curve in that it stresses the continuing activity of nonsplenic sources which we have already (1950) noted as increasing with increased antigenic stimulation. It is a probable curve independent of the stimulation of either the operation (see operative controls) or the removal of the inhibitory action of the spleen which acts after continued antigenic stimulation (see later discussion). Thus, according to figure 7, nonsplenic antibody in intact rabbits reaches a peak titer of 1:10,500 on the 15th day, which is only a third as high as that reached in splenectomized rabbits (1:30,700) on the same day. (This amount of nonsplenic antibody would be even less if a longer half life of splenic antibody had been used.) Nonsplenic antibody diminishes slowly after the 15th day. In other words, more is being continually formed.

The remarkable finding observed in the rabbits of experiment 4 is the fact that serum antibody disappeared from the blood, even though antigen was being injected, at a rate not significantly slower than that found in animals receiving a single injection of the same size. This disappearance cannot be due to the antigen injections because no change in downward slope was generally noted when injections of antigen were stopped during an antibody decline. In some repeatedly injected rabbits, the antibody curves were similar to those in singly injected animals, except that the titer reached a higher peak. This is notably true in rabbits 167 and 169 (figure 4B and C). In rabbit

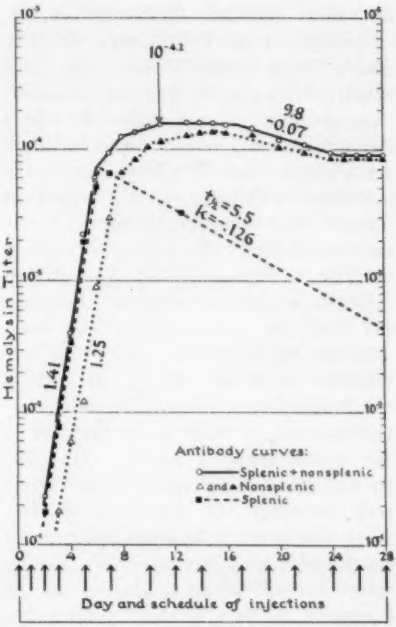


FIG. 7.—Mean daily hemolysin curve for 28 days from the 14 repeatedly injected intact rabbits of series 7 and the hypothetical separation of this curve into splenic and nonsplenic antibody. It is assumed that splenic antibody equals the difference between the mean daily titer of the intact (series 7) and splenectomized (series 8) rabbits through the 7th day (when the difference in titer between the two series was greatest) and that it stops being formed on the 7th day and disappears with a half life of 5.5 days. It is further assumed that nonsplenic antibody is the mean daily titer of the splenectomized rabbits (series 8) through the 7th day and thereafter equals the difference between the mean daily titer in the intact rabbits (series 7) and the hypothetical residual splenic antibody after the 7th day. Solid data points represent actual mean titers: open data points were calculated as indicated.

Splenic antibody appears sooner, is formed somewhat faster than nonsplenic antibody, but nonsplenic antibody is formed for a longer time

169, hemolysin reached a peak titer on the 6th day, disappeared for 37 days with an apparent half life of 12 days

and, after a plateau of 17 days, resumed about the same rate for 38 more days. In rabbit 167, the decline from peak titer on the 12th day continued almost uninterruptedly for 47 days with an apparent half life of 11 days.

Considerable evidence indicates that the spleen becomes relatively less important as antigenic stimulation is increased. Thus, in experiments 1 and 2 involving a ten-fold difference in the amount of antigen injected, peak titer in the intact animals was approximately the same but because of splenectomy dropped to 11% after the smaller amount and only dropped to 24% after the larger amount. Although peak titers in the two splenectomized groups were not significantly different, the splenectomized animals continued to form antibody at a higher rate for a longer time after receiving the larger dose. In 11 of 14 rabbits in series 4, peak titer was maintained for 5 to 20 days, whereas in the rabbits of series 2, antibody rapidly declined from peak titer in 5 of the 7 rabbits ($P = 0.026$). We have already presented reasons for believing that the multiple injections of antigen stimulated the spleen to form more antibody but stimulated nonsplenic sources to a greater extent.

These comparisons hold for the first part of the antibody curve, from the induction period to peak serum titer when the spleen is most active. The amount of antibody from the spleen, however, even in singly injected animals, which accounts for 76 to 89% of the antibody at peak titer, is approximately the same as that produced during the low grade action of nonsplenic sites over a long period. For example, using a uniform rate of decay, $k = 0.126$ ($t_{\frac{1}{2}} = 5.5$ days), we have calculated that nonsplenic sites in a typical antibody curve over a period of 90 days after a single injection of 1 ml of 10% cells is almost identical to that formed by the spleen. Similarly, using the hypothetical separation of splenic from nonsplenic antibody shown in figure 7 for a period of 90 days, we have calculated that nonsplenic sites after repeated injections may form about 8 times the antibody formed by the spleen. The relatively low increase in the importance of nonsplenic antibody sources as a result of multiple injections follows from the fact that the mean rate of decline of nonsplenic antibody from the peak in repeatedly injected animals is faster than in singly injected animals. In other words, nonsplenic sites stop producing antibody sooner in the repeatedly injected animals.

For the antibody rise in the above calculations we used equation (9). For the antibody decline in the splenectomized animals in which the decline is slower than the assumed rate of antibody decay, the amount of antibody formed (s) is assumed to be the total amount decayed (b) less the actual loss from the serum ($a_0 - a_t$).

$$s = b - (a_0 - a_t). \tag{12}$$

The amount of antibody present at time t will be

$$a_t = a_0 e^{-k_3 t} \qquad \text{(cf equation 3)} \tag{13}$$

where k_3 is the rate of antibody decline and a_0 is the amount of antibody at the beginning of the decline and is also the peak titer of the initial rise (A_t of equation 3). The total amount of antibody decaying (b), both of a_0 and of any antibody formed after time o, will be proportional to the antibody present. Therefore,

$$\frac{db}{dt} = k_2(a_0 e^{-k_3 t}) \tag{14}$$

in which k_2 is the rate of decay and is assumed to be the same as in equation (5).

$$(15) \qquad b = -a_0 \frac{k_2}{k_3} (e^{-k_3 t} - 1)$$

$$(16) \qquad = a_0 \frac{k_2}{k_3} (1 - e^{-k_3 t}).$$

The actual loss of antibody $(a_0 - a_t)$ is

$$(17) \qquad a_0 - a_t = a_0(1 - e^{-k_3 t}).$$

The total amount formed (s) is

$$(18) \quad s_t = a_0 \frac{k_2}{k_3} (1 - e^{-k_3 t}) - a_0(1 - e^{-k_3 t})$$

(by substitution in equation 12)

$$(19) \qquad = a_0 \frac{k_2 - k_3}{k_3} (1 - e^{-k_3 t}).$$

Motohashi (1922) noted a decrease in the effectiveness of splenectomy as the amount of antigen is increased. Thus, the titer in his intact animals was about 25 times as much as in splenectomized ones with a single injection of 0.5 ml of 50% cells (2.5 times our largest single injection); the ratio fell to 6 times the titer in splenectomized animals with two injections of 2 ml of 50% cells within 10 hours (20 times our largest single injection); and the titers were essentially the same in intact and splenectomized rabbits with 4 injections of 2 ml of 50% cells within 8 days (40 times our largest single injection) and 4 injections of 5 ml of 50% cells within 36 hours (100 times our largest single injection). Motohashi did not report the reversal of the effects of splenectomy although the animals in his last two series received total amounts of antigen comparable to our experiments 3 and 4 in which the splenectomized animals gave higher titers. This discrepancy may be due to the spacing of the antigenic stimulations or to the inherent inaccuracies of titers determined visually with a 100% end point.

The most striking finding in the present work is the reversal of the relative importance of splenic and nonsplenic sites in forming antibody during the initial rise after single and multiple antigen injections. Splenectomy resulted in a decrease to 11% in peak titer after a single intravenous injection of 1.25 ml of 1% sheep cells and an increase to 264% after 10 similar injections (fig. 1). Similarly, it resulted in a decrease to 24% after a single injection of 1 ml of 10% and an increase to 242% after 17 or more injections of the same amount. Possible explanations for the reversal of the relative importance of splenic and nonsplenic sites will be considered later on page 166. Regardless of the cause, the response in splenectomized animals receiving intense immunization is striking. In rabbit 187, not only was the peak titer 1:106,000, but a titer over 1:10,000 was maintained for 57 days. Such results suggest that splenectomy combined with repeated immunization might be put to practical use in the production of rabbit antisheep hemolysin for complement fixation. The fact that the titer decreased while antigenic stimulation was being continued indicates that immunization need not be continued longer than three weeks with the amount of antigen we used.

The data and analysis so far presented have led to the working hypothesis that the spleen and certain other, possibly all, antibody-forming sites produce a certain amount of antibody and then pass into an inactive and, at times, a refractory phase during which they can form little or no antibody until regenerative processes occur in them. With limited antigenic stimulation, different sites vary in the speed at which they enter the inactive state. It is, however, possible that all would enter it simultaneously with an overwhelming antigenic stimulation. When a site is re-

sponsible for a large fraction of the accumulating serum antibody, which is true of the spleen at peak titer after a single antigen injection, the cessation of activity during the inactive phase initiates an essentially logarithmic decrease of total serum antibody until a slowly declining level is reached which represents the continuing activity of other sites. The speed at which a given site enters and recovers from the inactive phase probably depends upon interrelated factors such as: (1) rate of antibody formation, (2) availability of intermediates for antibody synthesis, and (3) presence of antigenic stimulation.

The relationship between availability of antigen in a site and the speed and duration of antibody formation must be complex. The spleen, although forming more antibody during continued antigenic stimulation (page 161), forms it at the same rate as after a single injection. Thus, the values of k for the first part of the antibody rise (when the spleen is chiefly active) are the same after single and repeated injections (cf series 3 and 7 in table 2). The additional antibody is due to a longer continued formation. This fact suggests a characteristic rate (k) for each site. It is more likely, however, that there is a direct relationship between the amount of antigen and the rate of antibody formed up to a limiting point which is always reached for the spleen but is only occasionally reached for other sites under the conditions of our experiments. Thus, the values of k in the singly injected splenectomized animals are significantly lower than their intact counterparts (cf series 1 with 2 and 3 with 4 in table 2), whereas after frequent injections there is no significant difference between splenectomized and intact animals (of series 7 with 8 in table 2).

The spleen probably reaches its inactive phase first after a single antigenic stimulation because (1) it removes most of the antigen from the blood stream and (2) forms antibody at by far the highest rate. We have no quantitative evidence on the first point, but we know the spleen removes relatively large quantities of red cell antigen in the dog (Luckhardt and Becht, 1911) and removes the highest number of malaria-infected red cells per unit volume in monkeys (Taliaferro and Cannon, 1936, and review in Taliaferro and Mulligan, 1937). The high value of k for the spleen is indicated by the higher value of k in intact than in splenectomized animals. Furthermore, the high rate of antibody synthesis would lead to a quicker exhaustion of antigen and anabolic intermediates, both of which probably become limiting factors to varying extents at different times in the process of immunization (cf our earlier work, 1950).

For the present, we have made a distinction between the inactive phase during which a site resumes antibody formation upon further antigenic stimulation and the refractory phase during which antibody formation is not resumed in spite of continuous antigenic stimulation.

The reversal of antigen and anabolic intermediates as limiting factors because of their availability may be illustrated from our data. Lack of antigen, on the one hand, seems to be in part the limiting factor when the spleen stops forming antibody at peak titer after a single injection of antigen, because the spleen forms more antibody when antigen is repeatedly injected as in series 7. The absence of anabolic intermediates, on the other hand, seems to be in part the limiting factor when the spleen and other sites stop forming antibody in experiment 4 when antigen is being constantly supplied. The anabolic inter-

mediates may possibly be formed by enzymes, such as suggested by Burnet and Fenner (1949), or they may be the antibody precursors suggested by Green and Anker (1951 and personal communication). If these intermediates are derived from some stage in the synthesis of serum globulin, it is logical that they should be exhausted during such rapid antibody synthesis as occurs in the spleen.

The following facts reported in this and our earlier paper (1950) fit well into the foregoing hypothesis: Single injections of sheep cells, ranging from 1.25 ml of 0.1% to 80 times this amount, result in the spleen forming from 76% to 89% of the antibody at peak titer. The larger the antigen dose, the greater is the stimulation of nonsplenic sources. This conclusion is based upon the fact that splenectomy is less effective (Motohashi, 1922; our work, 1950; and fig. 1) and antibody disappearance is slower when the larger single antigen injection is given (our work, 1950). Multiple antigen injections ranging from 1.25 ml of 1% for 10 times during 26 days to 1 ml of 10% for 34 times during 74 days are effective in stimulating the spleen but stimulate nonsplenic sources even more. This conclusion is indicated by the daily ratios of serum titers in intact and splenectomized rabbits and by the continued rise and high titers attained in repeatedly injected splenectomized rabbits. In a real sense, multiple injections carry further the stimulation of nonsplenic sources obtained with the large single injection. When antigen was given often, the spleen and one or more nonsplenic sites sometimes reached their refractory phase early and at times almost simultaneously. Evidence for this is supplied by (1) the early attainment of peak titers in some intact and splenectomized rabbits in experiment 4 and (2) the subsequent disappearance of antibody at a rate within the order of magnitude accepted for the half life of serum globulin.

We have no evidence as to why repeated antigen injections stimulate splenectomized animals to produce more antibody from nonsplenic sources than do intact animals from splenic and nonsplenic sources together. Our data also leave uncertain the extent to which the operation itself stimulates antibody production. The operative controls in series 10 suggest that a large part, but not all of the effect of splenectomy is due to nonspecific stimulation. Those in series 11 suggest that removal of the spleen is the sole important factor. Assuming that the increased titer results at least in part from removal of the spleen, several possible explanations suggest themselves. Splenectomy plus repeated antigen stimulation may cause a hyperplasia or hyperactivity of nonsplenic cells involved in antibody formation. If such proves to be the case, both factors must be necessary as splenectomy alone is often followed in time by a resumption of antibody-forming power but not, in our experience, by an increase in this power. The mechanism which inhibits the spleen may also inhibit other sites. This possibility seems unlikely as the inhibition could only be partial because nonsplenic sources continue to elaborate antibody in intact animals after the spleen has stopped forming it. We tentatively prefer and are studying a third possibility, viz., the spleen in animals receiving multiple antigen injections prevents antigenic stimulation of nonsplenic sites by removing antigen from the blood even though it is itself in a refractory state and cannot form antibody. Parenthetically, the possibility should be considered that repeated injections of foreign red cells may partially blockade the animals. If such

a blockade is operative, however, it must be uniquely localized to the spleen. Thus, we obtained lower hemolysin titers after repeated injections in intact than in splenectomized rabbits, whereas the depressing effect of reticulo-endothelial blockade by intravenous injections is usually more pronounced in splenectomized than in intact animals.

It should be kept in mind that this discussion can as yet be applied only to the rabbit receiving the specified amounts of particulate red cell antigen by the intravenous route. We have already seen that the amount of antigen profoundly changes the antibody curve and the effects of splenectomy. Many investigators believe that physiological reactions to a particulate antigen, such as we have used, are fundamentally different from those to a soluble antigen (cf Burnet and Fenner, 1949). Others have stressed the peculiar serological responses of the rabbit—although in regard to the nature of the antibody rather than to the dynamics of antibody formation. In addition, routes of injection other than the intravenous one would probably change the results in so far as they would keep antigen out of the blood and cause its storage in macrophages other than those in the spleen, liver and bone marrow which are the main filter organs of the blood. Finally, we are aware of the danger of assuming a simple additive relationship between splenic and nonsplenic antibody sources. This idea has the advantage of being simple and our earlier work (1950 and 1950b) indicates that the spleen actually forms antibody and liberates it into the blood stream. Nevertheless, there are other possibilities involving the formation of intermediates at one site with the final synthesis elsewhere and/or the intersite stimulation or inhibition of synthetic processes.

CONCLUSIONS

1. During the first part of the antibody curve, i.e., the antibody rise to peak titer, antibody production increased faster in nonsplenic sites than in the spleen as antigenic stimulation was increased. Splenectomy reduced peak titer to 11% and 24% after a single injection of sheep red cells and increased it to 242% and 264% after multiple injections. Peak titers up to 1:106,000 were obtained in splenectomized rabbits repeatedly injected with a standardized red cell suspension equivalent to 1 ml of a 10% suspension/kg rabbit. Peak titers were 3 to 6.4 times higher in intact rabbits receiving repeated antigen injections than in singly injected ones; whereas they were 64 to 74 times higher in repeatedly injected splenectomized rabbits than in singly injected splenectomized ones. Over a long period, nonsplenic sources produced as much as or more antibody than the spleen. Thus, during the first 90 days, taking into consideration metabolic decay, it was estimated that after a single injection of 1 ml of 10% red cells nonsplenic sites formed as much antibody as the spleen, whereas after repeated injections of the same amount they formed 8 times as much.

2. Control operative procedures (removal of a testis via laparotomy, a kidney or the appendix) significantly stimulated antibody formation after one injection in otherwise intact animals. They also caused the production of as much total antibody as splenectomy in series 10 after 10 antigen injections but not in series 11 after more and larger antigen injections. In series 10, however, the operation did not stimulate nonsplenic sites as much as splenectomy.

3. The rise and decline of hemolysin in intact rabbits repeatedly injected

with sheep red cells fit a series of discontinuous curves described by the "compound interest" or first order reaction equation in which the constant of increment differs, as previously published for singly injected rabbits. The rates were essentially similar after single and multiple antigen injections but after multiple injections the titer began to rise sooner, reached a higher peak later and was maintained at a higher level for a longer time.

4. The data indicate that the spleen and certain other, possibly all, antibody-producing sites form a certain amount of antibody and then pass into an inactive and, at times, a refractory phase during which they can form little or no antibody until regenerative processes occur. The inactive phase of the spleen after one antigenic stimulation could be terminated by further antigen injections but eventually a refractory phase developed and continued in spite of repeated antigenic stimulation.

5. When a site is responsible for a large fraction of the accumulating serum antibody, its cessation of activity is followed by an essentially logarithmic decrease of serum antibody until the slowly declining level maintained by other sites is reached. Different sites usually vary in the speed at which they enter the inactive phase, and recovery probably depends upon such interrelated factors as rate of antibody formation, availability of intermediates for antibody synthesis and continued antigenic stimulation.

6. All titers were determined as 50% units read from plots, according to von Krogh's equation, of the amount of serum against the degree of lysis determined photometrically. Antigen (sheep red blood cells) for injection and testing was standardized by the photometric determination of hemoglobin liberated by distilled water lysis.

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THE EFFECT OF PENICILLIN ON TYPHOID ANTIBODY PRODUCTION

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In the course of a study on the effect of dietary factors on antibody production in man[1,2] we encountered a certain number of patients whose typhoid agglutinin producing ability fell short of expectation. Since many of these patients received penicillin as part of their therapeutic regimen, it was necessary to evaluate a possible effect of penicillin per se upon antibody producing mechanisms. A survey of the literature revealed reports of a lowering of antibody response in infectious disease following penicillin administration.

Skinses and Woolridge[3] indicated that prompt penicillin therapy depresses agglutinin antibody production as compared to delayed therapy, and that, theoretically, it may be advantageous to withhold such treatment until normal humoral defense mechanisms could be stimulated by the involved pathogens. Loge and Kilbourne[4] found in streptococcal pharyngitis that interference with antibody production as measured by the antistreptolysin titer was less marked in patients treated with large daily doses of penicillin than in those treated with small intermittent doses. In patients ill with scarlet fever Weinstein and Tsao[5] also found that continued treatment with penicillin resulted in a decrease in the ability to form antistreptolysin. Rantz et al,[6] determining antistreptolysin and antifibrinolysin formation following salicylate, sulfadiazine, and/or penicillin therapy in hemolytic streptococcal sore throat, reported that a short course of penicillin failed to diminish antibody response, whereas penicillin administration over a longer period had a deprecatory effect. Jordon[7] noted in 44 patients

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with pneumococcal pneumonia treated with penicillin that the antibody response was comparable to that occurring after a spontaneous recovery or recovery following treatment with sulfonamides.

According to Shwartzman,[8] in the absence of antibiotic therapy recurrent nasopharyngeal infections are never produced by the same type of organism, and prompt penicillin therapy is likely to prevent antibody formation with recurrent infection produced by the same type. Weinstein et al[9] noted in cases of pharyngitis due to beta hemolytic streptococci treated with penicillin at three hour intervals, that there was a marked reduction in the ability of these patients to produce significant antistreptolysin and/or antistreptokinase titers. The ability to produce adequate amounts of these immune bodies was increased considerably by lengthening the interval of penicillin administration to 8 hours. This was particularly true in the patients receiving oral penicillin. The authors expressed the opinion that the longer persistence of organisms in the pharynx on such courses of therapy might account for this increased antibody formation. Harrison,[10] comparing the effect of penicillin and sulfonamide drugs on the immune response of rabbits to pneumococcus infection, also noted almost a complete lack of agglutinin response when penicillin was administered early. He suggested that natural immunity in conjunction with the use of the drug eliminated the infection before antibody production was stimulated.

It is conceivable that penicillin may have a direct effect on antibody producing mechanisms. Hence we investigated the problem of differentiating whether penicillin has a direct effect on antibody production, or whether this depressing effect was mediated through the commonly accepted mechanism of the early abolition of the antigenic stimulus.

The following criteria for the selection of cases was established: first, that penicillin be used; second, that there be an absence of acute infectious disease; third, that the biologic action of the vaccine be not modified by the penicillin;* and finally, that satisfactory controls be obtainable.

MATERIALS AND METHODS

A total of 28 patients were studied; 14 patients were treated with penicillin, and the remaining 14 patients served as controls. The group receiving penicillin included 9 patients with paresis, two with cerebrovascular accidents, and three with miscellaneous lesions. The control group included 6 patients with paresis, two with cerebrovascular accidents, and 6 with miscellaneous lesions (see table 1).

To exclude the possibility that the kind, dose, or mode of administration of penicillin or typhoid vaccine might in some way alter the antibody response, several dosage schemes in regard to both penicillin and typhoid antigen were studied. All paretic patients received at weekly intervals 0.2 cc of typhoid vaccine intracutaneously for a total of 2 injections. The vaccine contained approximately one-half billion organisms per cc. Nine of the paretics received one intramuscular injection of a crystalline peanut oil, aluminum monostearate preparation of penicillin,† containing 2.4 million units. The injection of penicillin was given simultaneously with the first inoculation of typhoid antigen. Six of the paretics served

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* Typhoid vaccine, to our knowledge, is not affected by penicillin.

† Pazillin Procaine Penicillin G (Sharpe and Dohme).

TABLE 1.—*Typhoid antibody agglutination titer.*

Disease	Penicillin treated				Control		
	Penicillin dose	Patient's name	Titer dilution at end of		Patient's name	Titer dilution at end of	
			7 days	14 days		7 days	14 days
Paresis	2.4 mill. units—one injection	Seve.	0	1:640	Care.	1:320	1:2,560
		Fore.	1:40	1:2,560	Coar.*	1:10,240	1:40,960
		Smit.	1:5,120	1:10,240	McCl.	1:2,560	1:10,240
		Simm.	0	0	Ford.	1:1,280	1:2,560
		Fore.*	1:10,240	1:20,480	Pitm.	1:320	1:10,240
		Brow.	1:1,280	1:2,560	Rich.	1:640	1:5,120
		Bran.	1:1,280	1:10,240			
		Scot.	0	0			
		Scam.	1:80	1:1,280			
Cerebro-vascular accidents	300,000 units o.d. for 14 days	Craw.	0	1:160	Thoma.	1:320	1:320
		Marr.	1:80	1:160	Thomp.	1:40	1:160
Misc. Group	100,000 units o.d. for 14 days	Grei. (leg ulcer)	0	1:40	Guin. (arthritis)	1:160	1:1,280
		Tell. (neuro-dermatitis)	0	1:320	McCle. (leg ulcer)	1:40	1:160
					Reil. (neuritis)	1:80	1:320
	300,000 units o.d. for 14 days	Lenn. (pulmonary tuberculosis)	0	1:640	McCld. (leg ulcer)	1:320	1:1,280
					Mage. (leg ulcer)	1:40	1:640
					Breu. (arterio-sclerotic gangrene leg)	0	1:40

Titers expressed as 0 represent antibody response less than 1:20, the lowest dilution used. Use of either 0 or 1:20 is of no consequence in the calculation of significant difference.
* Patients not included in table 2.

as controls and were similar to the experimental group, except that they received weekly injections of tryparsamide or bismuth.

All nonparetic patients in both treated and control groups received at weekly intervals 0.5 cc typhoid antigen subcutaneously, for a total of three injections. Five of these patients received either a course of 300,000 units of aqueous (procaine) penicillin or a course of 100,000 units of crystalline penicillin daily for 14 days. The initial dose of typhoid vaccine was administered simultaneously with the first injection of penicillin. Eight of the nonparetic control patients received no antibiotics but were given nonspecific medication, including sedatives, analgesics, cathartics, and local antiseptic dressings, which contained no antibiotic agent and which as far as is known, had no effect on antibody production.

Blood specimens were collected prior to the administration of penicillin in the treated group, prior to the injection of typhoid antigen in both groups, and at weekly intervals during the experimental period. Cases showing an initial antibody titer for *Salmonella typhosa* (flagellar "H" antigen type) were excluded from the study. All the patients in this study had serum protein and blood hemoglobin levels within normal limits.

The typhoid antibody agglutination titer was determined weekly, and the titer was read as the highest dilution in which a gross evidence of agglutination was seen. Methods used in this study were previously described.[1,2]

RESULTS

Table 1 summarizes the typhoid antibody agglutination titer developed one and two weeks after typhoid vaccine. This table also indicates the disease present in the studied cases and the dosage of penicillin administered.

Table 2 indicates that there is no significant difference* between the mean titer in the treated and control groups.

* No statistical difference as indicated by "T" tests.

TABLE 2.—*Comparison of the mean titer of the injected and control groups.*

	7 days		14 days	
	Penicillin	Control	Penicillin	Control
No. of cases	13	13	13	13
Mean titer	606.2	470.8	2,218.5	2,686.2
Range	0 to 5,120	0 to 2,560	0 to 10,240	40 to 10,240
Standard deviation	1,435.5	716.9	3,668	3,642.1

The standard deviation and wide range in each group is also noted. The antibody titer in two patients (one in the treated and one in the control group) was not included in calculating the mean titers of their respective groups; it is our opinion that such an overwhelming antibody response represented an anamnestic reaction.

Two patients treated with penicillin had no typhoid antibody production at the end of two weeks; however, no conclusion can be drawn from this small number of cases.

DISCUSSION

The importance of the human defense mechanism in addition to the use of specific drug therapy in producing complete recovery from infectious disease is generally appreciated. The significant relationship of the development of adequate antibodies to dietary factors has been previously noted.[1,2,11] The ideal drug would then necessarily be one which had a specific effective action on the antigen and which at the same time did not interfere with normal immune reactions. Although it is believed that the various chemotherapeutic and antibiotic agents have no direct effect on immune processes, proof by appropriate studies in man was not noted in a review of the literature. The implication that penicillin may paradoxically hinder the body's mechanism of defense is of far reaching clinical significance. In the present study the antigenic stimulus was kept constant in both the treated and control groups, since the antibiotic used could not have acted on the antigen—the latter consisting of killed typhoid organisms. This permitted an evaluation of the possible direct effect of penicillin on antibody production. Our data suggest that penicillin per se does not inhibit antibody producing mechanisms as measured by typhoid agglutinin titer.

SUMMARY

Twenty-eight patients were studied for their ability to produce typhoid antibody titer after equal and adequate stimulation with typhoid vaccine. Fourteen of these patients were treated concurrently with penicillin.

There was no statistical difference between the mean antibody titer developed in the treated and control groups. The evidence is suggestive that penicillin per se does not appreciably affect the body's ability to produce agglutinins against typhoid antigen. The inhibitory effects of penicillin on antibody production referred to in the literature are probably on the basis of the early destruction of the pathogenic organisms.

11. Cannon, P. R., Chase, W. E. and Wissler, R. W. 1943, The Relationship of the Protein Reserves to Antibody Production. J. Immunol. **47**: 133–147.

A GRANULOSIS OF THE OMNIVOROUS LOOPER, *SABULODES CABERATA* GUENÉE

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In the course of rearing the omnivorous looper, *Sabulodes caberata* Guenée, a virus disease was noted in the larvae of this insect by Irvin M. Hall, of this laboratory. The affected insects were a part of the first generation that had been maintained in the laboratory, the adults of the previous generation having been collected in El Cerrito, California. The malady, the first virus disease to be reported in this insect, proved to be fairly typical of that group of virus infections known as granuloses. It is the purpose of the present paper to describe the disease, its histopathology, and its causative virus.

MATERIALS AND METHODS

For histological study slides were prepared by fixing diseased and normal insects in either Bouin's or Carnoy's fluid, dehydrating in dioxane, sectioning, and staining with Mallory's triple stain.

In preparing the virus for electron microscopy, essentially the same technique was used as that published by Steinhaus et al (1949). Tissue from infected insects was ground in a mortar, after which the granules that characterize this group of diseases were separated from tissue debris by centrifugation. The granules were then suspended in 0.1 *M* sodium carbonate-0.05 *M* sodium chloride for one hour. The virus particles thus freed of their granular covering were washed and concentrated by two more centrifugations of one-half hour each at 13,000 r.p.m. (20,000 g). The final sediment (fig. 2D) was mounted in the usual manner for electron microscopy. During this process, samples of the partly purified suspension of granules were withdrawn before the addition of the alkali and mounted on electron microscope screens for study of the intact granules (fig. 2A). Partial breakdown of the granules (fig. 2B) was obtained by pipetting 0.01 *M* sodium hydroxide directly on the screens after the droplet of granule suspension had dried. After 5 minutes, the sodium hydroxide was removed, the screens washed in distilled water and allowed to dry. All electron microscope preparations were shadowed with palladium.

CHARACTERISTICS OF THE DISEASE

A larva of *Sabulodes caberata* assumes a somewhat mottled appearance when affected by this disease. The normally green integument becomes blotched with white. A chalky white area becomes visible along the ventral side of the abdomen in the later stages of the infection. The latter effect results from the unnatural white appearance of the fat body showing through the translucent integument. Upon dissection, the fat tissue of the infected larva has an opaque white appearance, whereas the corresponding tissue of a healthy larva is a faint straw color and nearly transparent. After death, the diseased larva often turns a golden brown color.

Larvae are easily infected by feeding them sprigs of alfalfa dipped in a water suspension of infectious material obtained by grinding the carcasses of insects which have died of the disease. Of 60 larvae reared individually and 150 reared in groups of 5, 100% were infected by this method. The time from

the beginning of infection to the death of the insect varies considerably, with most of the mortality occurring in from 10 to 20 days after infection.

In cross-infectivity tests the following insects were found to be insusceptible: the buckeye caterpillar, *Junonia coenia* Hübner; the variegated cutworm, *Peridroma margaritosa* (Haworth); and the salt marsh caterpillar, *Estigmene acraea* (Drury). These three species are the only other insects occurring in California in which granuloses are now known to occur. Similarly, no infection could be produced in *Sabulodes caberata* Guenée when fed the viruses from the other three insects.

Histopathology

The only tissue in which we have found any pathological changes during the course of the disease is the adipose tissue. The gross appearance of this tissue has been described above. Under the microscope, diseased fat cells appear more nearly opaque than normal fat cells and have a brownish coloration when seen in a fresh, unstained preparation. Heavy concentrations of free granules in a water suspension show the same brown tint. Spherical vesicles containing many granules agitated by brownian movement are sometimes seen in water suspensions of fresh material. These spheres have been seen to form within an infected fat cell and to be discharged through the periphery of the cell. Just what these spherical structures are is not clear. Although this phenomenon is observed not infrequently in fresh material, nothing has been seen in stained sections of diseased tissue which would appear to be related in any way to this process. Similar phenomena have been reported by Paillot (1937) and by Bergold (1948) and have been observed numerous times in this laboratory during studies of other granuloses.

In stained sections, the first noticeable evidence of the disease is an enlargement of the nuclei of infected fat cells. At the same time, the nuclear material seems to break up and condense into brightly staining, irregularly shaped masses. These may appear scattered at random throughout the nuclear area or they may take a position forming a complete or incomplete ring of particles just inside the nuclear membrane. Sometimes the degenerating nuclear material may form a continuous ring or partial ring rather than breaking up into a number of separate bodies (fig. 1Bd). Filling the remaining space of the nucleus are the granules (fig. 1Bc). It is only rare that these may be seen as separate bodies in a stained section. Usually they stain in a more homogenous manner, appearing as a mass having a vaguely granular appearance. At this stage the cytoplasm of the host cell appears perfectly normal. As the disease progresses, the nucleus continues to increase in size until it occupies a large portion of the cell. At the same time the entire cell increases in size. This appears to be a result of the hypertrophy of the nucleus, for the cytoplasm seems to be reduced in volume. Vacuoles filled with oil, which normally fill a large part of the cytoplasmic region of the healthy fat cell, are not as common at this stage of the infection. In the final stage of the process, it is no longer possible to distinguish between nucleus and cytoplasm. Either the nucleus has hypertrophied to the point that it occupies the entire cell or the nuclear membrane has ruptured or disappeared allowing the nuclear and cytoplasmic elements to mingle. The second possibility appears to be the one indicated by the sections studied. The cell in an advanced stage of disease consists of a cell membrane surrounding a mass of granules through which fragments of nuclear material are

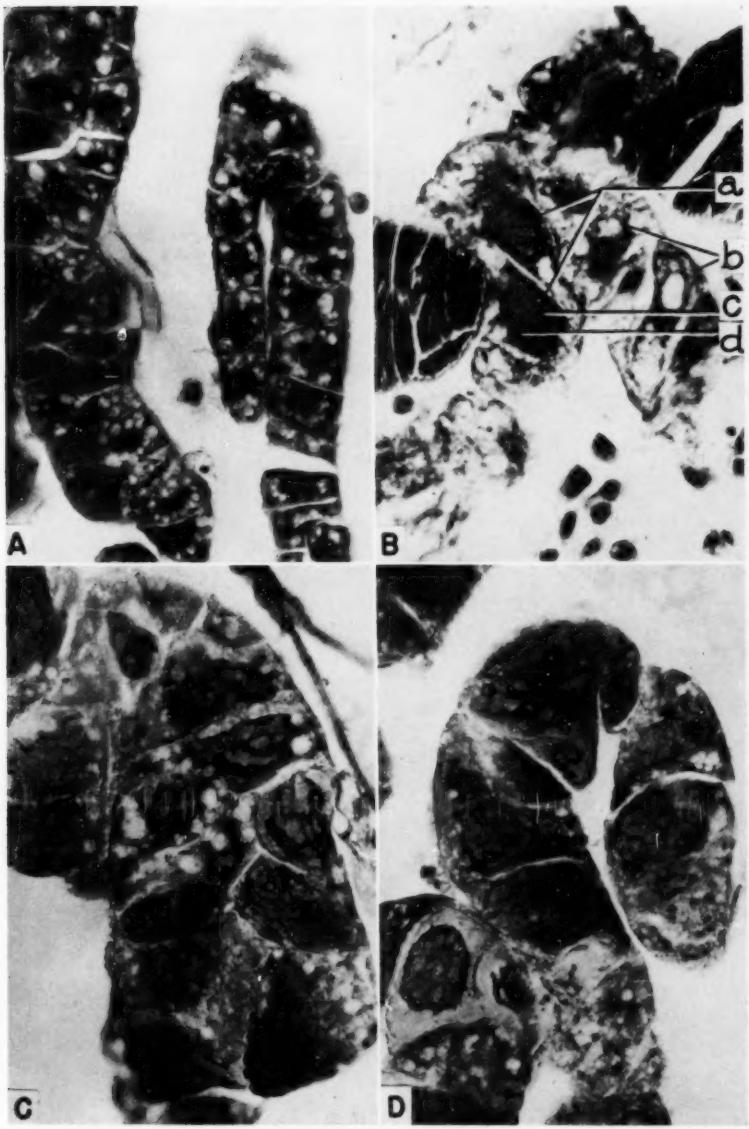

FIG. 1.—Photomicrographs of adipose tissue of *Sabulodes caberata*. A. Healthy adipose tissue. B. Tissue from insect in early stage of granulosis; a, infected fat cells; b, normal-appearing fat cells; c, mass of granules in center of nucleus; d, chromatin material at periphery of nucleus. C. and D. Tissue from insect in late stage of infection. Magnifications: A and B, 650×; C and D, 475×.

scattered. A darker-staining network usually appears throughout the mass of granules (fig. 1C). What the latter represents is not known.

All cells within a given tissue are not affected simultaneously. If the disease has not progressed to its last stages, it is quite usual to find an apparently healthy fat cell situated next to one that has a greatly hypertophied nucleus (fig. 1B). It appears, however, that eventually all fat cells are affected.

The question of the origin of the granules is one that does not appear to be satisfactorily solved. Since the known granuloses are quite similar with respect to most of their characteristics, it seems reasonable to believe that the origin of the inclusion bodies is probably the same in all diseases of this type. Yet, published descriptions of granuloses are not in agreement on the question of whether the inclusion bodies first appear in the nucleus or the cytoplasm of the host cell. In publishing the first description of a disease of this type, Paillot (1926) stated that the inclusion bodies (which he believed to be the etiological agent) "multiplied" in the cytoplasm of the cells of the host, *Pieris brassicae* (Linn.). Later the same investigator (1936) described two granuloses of *Euxoa segetum* Schiff., in one of which he found granules appearing first in the cytoplasm, while in the other he found that granules "se multiplient surtout à l'intérieur du noyau . . . mais également dans la couche cytoplasmique." A third granulosis of the same insect was described by Paillot (1937) as having granules appearing in the nucleus. Bergold (1948) found the granules of *Cacoecia murinana* Hübner appearing in the cytoplasm. Steinhaus and Thompson (1949), in describing the granulosis of *Junonia coenia*, say: "The granules possibly originate within the nucleus and sooner or later accumulate in the cytoplasm." In the disease of *Sabulodes caberata*, it appears that the granules form in the nucleus. This is evident only in cells in which the infection has not advanced too far. In such cells, what appears in a stained section to be a mass of granules can be seen within the nuclear membrane, whereas no granules can be distinguished in the cytoplasm. At a later stage, however, nucleus and cytoplasm do not appear as separate entities, and the cell appears to be filled with granules and nuclear remains. In such a cell the granules give the appearance of being in the cytoplasm, since they occupy the space in which the cytoplasm is normally found. It is possible that some reports of granules which arise in the cytoplasm may have resulted from observations made after the infection had progressed too far to distinguish between nucleus and cytoplasm. At least, in the present case it appears that the inclusion bodies arise in the nucleus.

Inclusion bodies and virus

The granules are of such a size that they are visible—although none too distinctly—with the higher powers of the light microscope. In a water suspension they appear as tiny dots rapidly dancing with brownian movement. A heavy concentration of the granules has a brownish coloration that is most readily distinguished under a low power objective. When observed with dark field and an oil immersion lens, the bodies are easily seen.

When studied with the electron microscope the granules are seen to be roughly ovoid bodies which average 345 mμ in length by 170 mμ in width (fig. 2A). They vary considerably in both size and shape. Their variability and the manner in which granules appear to adhere to each other seem to indicate that the material of the gran-

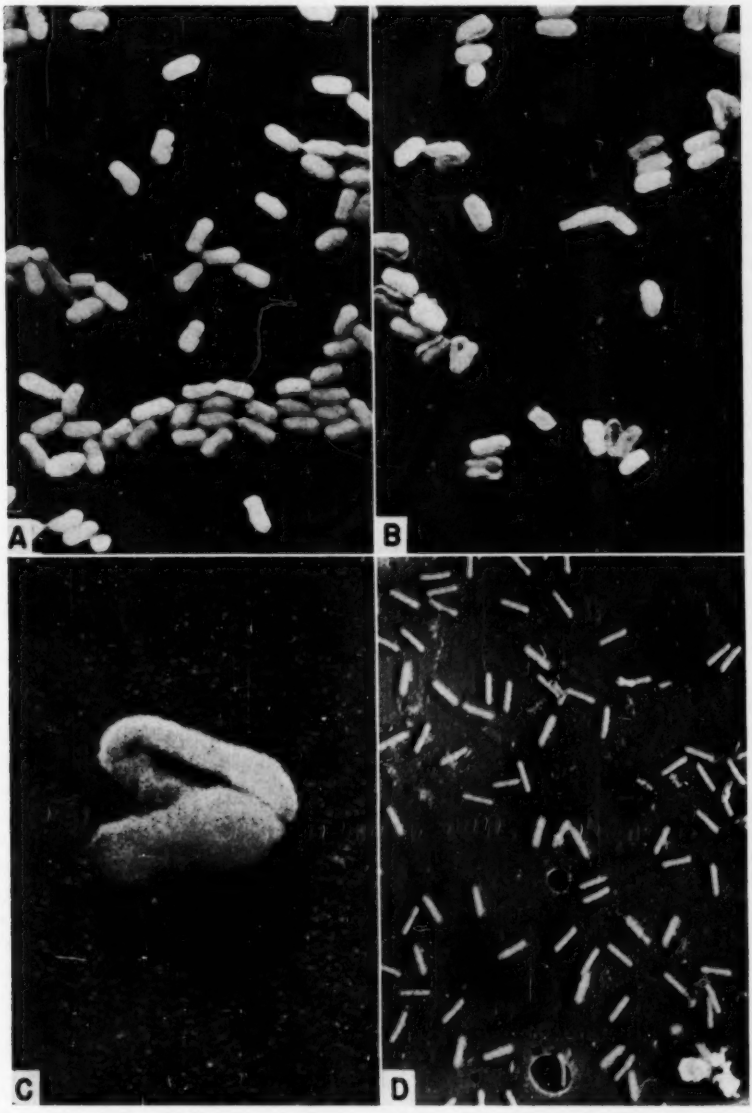

FIG. 2.—Electron micrographs of granules and virus particles. Shadowed with palladium. A. Untreated granules. B. and C. Granules after treatment with sodium hydroxide. D. Virus particles, some of which appear to be enclosed in a membrane. Magnifications: A and B, 18,900×; C, 100,000×; D, 21,400×.

ules may be rather soft and viscous or that, at least, the exterior of the granule is covered by such a substance. Bizarre forms such as that shown in figure 2A are seen not infrequently. Similar forms have been described in other granuloses (Bergold, 1948; Steinhaus et al, 1949).

When treated with sodium carbonate, the granules dissolve leaving the freed virus particles (fig. 2D). In the case of the granulosis of *Cacoecia murinana*, Bergold (1948) states that there is one virus rod within each granule. Such is apparently the case in this disease. The granules treated with sodium hydroxide exhibit a partial breakdown which often takes the form of a longitudinal splitting (fig. 2B and 2C). Such split granules show what appears to be an internal cavity having approximately the size and shape of a virus rod.

The virus particles are rod-shaped structures similar to other granulosis viruses described. Their average size is 275 mμ long by 65 mμ wide. There is a noticeable variation in the length of the particles, ranging from 245 to 300 mμ.

Some of the rods appear to be surrounded by a membrane (fig. 2D). Bergold (1950) has indicated that this is the case with the granulosis virus of *Cacoecia murinana*. What appear to be empty, collapsed membranes can be seen in some preparations (fig. 2B). Roughly spherical bodies are sometimes seen in preparations of the virus. These have about the same opacity to the electron beam as typical virus rods have, and may represent a different stage of the virus. In some cases these appear to be a rod folded in the middle and surrounded by a membrane. Similar forms have been seen by the authors from time to time during the course of studies of several other insect viruses. Reports of such forms have been made by Bergold (1950) and by Wasser and Steinhaus (1951).

In light of the cross-infectivity attempts, it appears that the virus exhibits the same high degree of host specificity that has been found to exist among other insect viruses and the authors feel justified in considering this virus distinct from others previously reported. According to the classification of insect viruses proposed by Steinhaus (1949a, b), this virus would be placed in the genus *Bergoldia* by virtue of its type of inclusion body. The authors propose the name *Bergoldia nosodes* n. sp. (Greek *nosodes*, making sick) for the virus herein described. A preparation of the type strain has been deposited in the American Type Culture Collection.

SUMMARY

A granulosis disease of the omnivorous looper, *Sabulodes caberata* Cuenée, is described. The characteristics of the disease and its causative virus are similar to those of other granuloses but the virus is not identical with any previously described. The only host tissue known to be affected is the adipose tissue. Ovoid inclusion bodies 345 mμ by 170 mμ are found in the nucleus of the infected cell. The nucleus hypertrophies greatly and eventually the nuclear membrane appears to break down. The chromatin material of the nucleus degenerates into irregularly shaped, densely staining masses. Each inclusion body contains a virus particle measuring 275 mμ by 65 mμ. The name *Bergoldia nosodes* n. sp. is proposed for the virus.

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STAPHYLOCOCCAL ENTEROTOXIN: PRODUCTION METHODS

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An investigation has been initiated with the objective of gaining more fundamental knowledge of the nature of staphylococcal enterotoxin. The purpose of this report is to describe culture methods which have been utilized for producing large quantities of enterotoxin, a necessary step preliminary to purification studies. A thorough study of the factors affecting formation of the toxin by staphylococci was not possible due to the lack of a quantitative assay method. The immediate goal was to devise practical methods for producing large quantities of enterotoxin in a dialyzable medium.

Three methods which we have employed for producing 10- to 15-liter lots of crude toxin in an enzymatic casein-digest medium are described first. This is followed by a less detailed description of modifications of these and other culture techniques which have yielded enterotoxin.

MATERIALS AND METHODS

Stock cultures of different toxigenic capacities have been employed (for description see Surgalla[1]). The strains used in most of the work (196 and a variant, 196E) produce enterotoxin and both alpha- and beta-hemolysins.

The medium used for the most part is similar to that described by Favorite and Hammon.[2] The composition per liter is: amigen (pancreatic digest of casein, Mead Johnson) 15 g, nicotinic acid 0.00123 g, thiamin hydrochloride 0.00005 g, and glucose (anhydrous) 2.25 g. The medium is adjusted to pH 7.6 before sterilization in the autoclave. Modifications and other mediums used are described with the culture methods.

Tests for the presence of enterotoxin were made by feeding culture supernatant fluid (usually 50 ml) to young monkeys (*Macaca mulatta*) by stomach tube. Animals were observed for 5 hours after feeding, and vomiting was accepted as a positive criterion for enterotoxin. Tests for hemolysins were made as described previously.[1]

PRODUCTION METHODS

Shallow cultures.—Two hundred ml of medium were placed in a Roux bottle, sterilized in the autoclave, and inoculated with 0.5 ml of culture (18 to 24 hours). Fifty of these bottles (10 L of culture) were placed in an autoclave which had been converted into an incubator and used at 36 to 38 C. The incubator was partially evacuated, and carbon dioxide was introduced manometrically to replace approximately 20% of the air. The incubator was sealed, and cultures were incubated for approximately three days.

Deep cultures in turning bottles.—Ten L of medium were placed in a 12-gallon pyrex bottle and sterilized in the autoclave. Inoculums ranging from 2 to 900 ml of 18 to 24-hour culture have been used, although 10 to 70 ml were usually employed. The bottle was sealed with a rubber stopper through which extended a piece of glass tubing plugged with cotton. The culture bottle was partially evacuated, and carbon dioxide was introduced to replace approximately 20% of the air. The glass tube

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1. Surgalla, M. J. 1947, J. Infect. Dis. **81**: 97–111.
2. Favorite, G. O. and Hammon, W. M. 1941, J. Bact. **41**: 305–316.

was sealed in a flame and the bottle placed in the 37 C incubator on rollers which rotated the bottle at a rate of three times per hour. Cultures were incubated for 2 to 5 days.

Over 2000 L of crude toxins have been produced by a combination of the above two methods. In the preparation of large pools for chemical studies[3] not all of the individual lots of crude toxin were tested in monkeys; and of those tested not all were positive. Of 94 lots of crude toxin tested (usually in two monkeys each), 46 lots or 49% were positive for enterotoxin. This figure would vary, depending on the current supply of fresh monkeys, since negative feeding tests with a limited number of animals of varying susceptibility do not necessarily indicate the absence of enterotoxin in a sample.[4]

TABLE 1.—*Aerated cultures which caused vomiting when fed to monkeys.*

Experiment	Medium	Liters of culture	Bottle size (L)	Inoculum %	Glucose %	Hours of incubation	Aeration			Final pH	Hemolysin titers	
							Gas used	Rate l/hr	Orifice size		alpha	beta
1†	AA*	4	9	0.025	0.2	96	air	discon.*	S.G.*	6.6		
2†	VIB*	2.6	10	0.04	0	120	CO_2-air*	discon.	S.G.	7.1		
3	1.5% Amigen*	5	9	0.02	0.2	48	air	discon.	S.G.	6.2		
4	1.5% Amigen	1	2	0.05	0.2	72	air	12	1.4 mm	7.2	16	64
5	1.5% Amigen	1	2	1.0	0	20	CO_2-O_2	7	4.6 mm	7.2	512	64
6	1.5% Amigen	1	2	7.0	0	46	air	13	4.6 mm	8.1	0	128
7	3% Amigen	7	9	1.8	0.82‡	66	air	discon.	S.G.	7.2	16	256
8	2% AHC*	15	20	0.2	1.04‡	67	air	30	S.G.	7.8	16	256

Initial pH of mediums before autoclaving was 7.6. Incubation temperature was 37–38 C. Inoculation was made with cultures in amigen medium less than 24 hours old.

* Key: AA = amino acid medium containing 6 amino acids. Discon. = discontinuous aeration. S.G. = sintered glass sparger. VIB = veal infusion broth. CO_2-air = mixture of approximately 20% CO_2 + 80% air. CO_2-O_2 = mixture of 20% CO_2 + 80% O_2. AHC = acid hydrolyzed casein. Amigen = pancreatic digest of casein (Mead Johnson).

† Enterotoxin detected only after concentration of crude preparation.

‡ Glucose and sodium hydroxide added at intervals during incubation.

Deep aerated cultures.—Production data for 8 lots of enterotoxin from aerated cultures are presented in table 1. Only the 15 L culture is described here in detail. Fifteen liters of medium were placed in a 5-gallon pyrex bottle and sterilized in the autoclave. After inoculation of the medium, the cotton stopper was replaced with a rubber stopper penetrated in several places by glass (or stainless steel) tubes connected by rubber tubing to provide several closed siphon systems. These lines provided for 1) introduction of glucose from a calibrated bottle containing 50% glucose; 2) introduction of sodium hydroxide; 3) removal of samples of culture; 4) aeration through a sintered glass sparger extending almost to the bottom of the bottle; 5) escape of air, and 6) introduction of an antifoam agent (lard oil) through a rubber vaccine-bottle stopper. Air was filtered through cotton before entering and after leaving the culture bottle. Ingoing air was bubbled through water at 37 C to avoid evaporation of culture medium. Outgoing air passed through a flowmeter. Temperature of incubation was 37 C.

The medium used in this experiment was prepared with acid hydrolyzed casein (adsorbed with charcoal to remove the dark color) used at a concentration of 2% based on original protein and containing 70 mg of phenol red indicator. The inoculum was 30 ml of a 6-hour culture. Aeration was with air at a rate of approximately 30 L per hour. Glucose and sodium hydroxide were

3. Bergdoll, M. S., Kadavy, J., Surgalla, M. J. and Dack, G. M. To be published.

4. Surgalla, M. J. et al. To be published.

added when the culture became acid. Samples taken before and after these additions were assayed for hemolysins and tested for pH using the Beckman pH meter. The samples ranged from pH 6.2 to 8.4. The total glucose added was about 1% during an incubation time of 67 hours. Addition of 4 ml of lard oil to the medium controlled foaming.

ADDITIONAL EXPERIMENTAL RESULTS

Roux bottle cultures.—Enterotoxin has been produced in culture mediums prepared from 1) veal infusion broth; 2) acid hydrolyzed casein, or 3) enzymatic casein-digest (amigen). Enterotoxin has demonstrated in amigen cultures produced under the following sets of conditions: 1) with or without added glucose, 2) with or without $M/30$ phosphate, and 3) incubation in an atmosphere with or without added carbon dioxide.

A pink color occasionally appears in cultures of strain 196E grown in an amigen medium. This color is associated with the precipitate upon saturation with ammonium sulfate but is lost during dialysis through #36/32 Visking casing. Specific conditions for its production have not been defined. It appears in Roux bottle cultures and sometimes in aerated deep cultures. Judging from gross observations it appears to reach a maximum of intensity at some time less than three days and then diminishes. We have never observed this pink color in cultures of the parent strain 196.

Kolle flask cultures.—Semisolid veal infusion agar has for many years been one of the best mediums known for the production of potent enterotoxin.[5,6] In these studies enterotoxic cultures have also been prepared on semisolid agar mediums prepared with 1) amigen medium, 2) a synthetic medium containing 15 amino acids, and 3) veal infusion broth dialysate.

When the agar in semisolid mediums was replaced by silica gel, the culture supernatant fluids sometimes gave negative tests for enterotoxin, while washings of the silica gel were positive.

Liquid veal infusion broth and amigen cultures in Kolle flasks contained enterotoxin. In general, amigen and veal infusion mediums have given comparable results when used under the same conditions.

Liquid cultures in turning bottles and flasks.—The routine method described earlier (10 L of culture in a 12-gallon bottle turning on rollers) is an adaptation from smaller scale methods used previously by other workers. Favorite and Hammon[2] produced toxin in 20 to 45 ml lots of hydrolyzed casein medium in 250 ml pyrex hexagonal nursing bottles with horizontal rotation during incubation. Similarly, toxin has been produced in 100 ml lots in 500 ml Florence flasks by Segalove[7] (using amigen medium) and Surgalla[1] (using amino acid mediums). In the present investigation toxin has been produced in 100 ml lots of veal infusion broth, in 500 to 10,000 ml lots of amigen medium, and in 10,000 ml lots of acid hydrolyzed casein medium.

Hemolysin production in aerated cultures.—Hemolysin titers obtained in several aerated cultures are given in table 2. The beneficial effect of carbon dioxide is apparent. (Although in these aerated cultures a mixture of 20% carbon dioxide plus 80% oxygen was used, similar effects on lysin titers were observed in shallow Roux bottle cultures when a mixture of 20% carbon dioxide plus 80% air was used.) In this set of experiments the role of pH in the in-

5. Dack, G. M. 1949, Food Poisoning, revised edition, Chicago, University of Chicago Press.
6. Dolman, C. E. 1943, Canad. Pub. Health J. **34**: 97–111; 205–235.
7. Segalove, M. 1947, J. Infect. Dis. **81**: 228–243.

hibiting effect of 0.5% glucose was not controlled. Optimum aeration methods for lysin production have not yet been determined. Slow aeration with large bubbles has been as effective as has faster aeration through sintered glass spargers. In three different experiments when one of two parallel culture bottles was shaken vigorously once daily, the alpha- and beta-hemolysins were either completely absent or greatly reduced in titer as compared with the undisturbed control bottles.

sorption on organic liquid emulsions (ether or chloroform).

Inhibitors of toxin-formation.—McLean[11] found that higher titers of alpha-hemolysin could be produced in broth if cellophane was first soaked in the broth. This beneficial effect of cellophane occurred whether it was removed before inoculation or remained during incubation. He suggested that the cellophane acted by adsorbing from the medium some substance which inhibited hemolysin production but not growth of

TABLE 2.—*Production of hemolysins in aerated cultures in pancreatic digest casein medium.**

Experiment no.	Strain	Liters of culture	Glucose %	Hours of incubation	Aeration rate l/hr	Gas used	Final pH	Hemolysin titers	
								alpha	beta
1	196E	1	0.5	46	15.6	air	5.2	0	4
		1	0	46	13.2	air	8.1	0	128
2	196E	1	0.5	46	11.4	CO_2-O_2†	5.2	0	4
		1	0	46	16.6	CO_2-O_2	7.5	256	256
3	196E	1	0.2	41	8.6	air	5.6	8	256
		1	0.2	41	8.4	CO_2-O_2	6.6	64	256
4	196E	1	0	20	7	CO_2-O_2	7.1	512	64
5	196E	1	0	29	13.4	CO_2-O_2	7.7	512	256
		7	0	29	27	CO_2-O_2	7.5	256	128
6	44	1	0	16	12.9	CO_2-O_2	6.9	256	8
7‡	J32A	1	0	18	18.8	CO_2-O_2	7.7	0	512
		1	0	18	15.4	air	8.7	0	32

* "Amigen," Mead Johnson.
† CO_2-O_2 = mixture of 20% carbon dioxide and 80% oxygen.
‡ Aeration was through a sintered glass sparger; all other cultures were aerated with large bubbles from an orifice of 4.6 mm diameter.

Casman[8] reported greater yields of alpha-hemolysin when a suitable carbon dioxide-oxygen gas mixture was passed through his veal-peptone broth cultures. Gladstone[9] reported that alpha-hemolysin produced in synthetic medium was easily destroyed by passing gases through the hemolysin, even when nitrogen (free from oxygen) was used. Mechanical shaking with a gas also inactivated the lysin. Johlin and Rigdon[10] reported rapid inactivation of hemolytic and dermonecrotic properties by adthe staphylococci. A similar effect was produced by silica gel. Kieselguhr and kaolin were effective but adsorbed the lysin if not removed before inoculation. Phosphate precipitates (produced during sterilization in the autoclave), agar, and filter paper were effective only if left in the culture after inoculation. Calcium sulphate, Norit, and sliced potatoes were ineffective. McIlwain,[12] working with simpler mediums, did not find any evidence of inhibitory substances in the mediums but attributed an inhibitory effect to degradation

8. Casman, E. P. 1940, J. Bact. **39**: 84.
9. Gladstone, G. P. 1938, Brit. J. Exper. Path. **19**: 208–226.
10. Johlin, J. M. and Rigdon, R. H. 1941, J. Immunol. **41**: 233–240.
11. McClean, D. 1937, J. Path. Bact. **44**: 47–70.
12. McIlwain, H. 1938, Brit. J. Exper. Path. **19**: 411–417.

products of glucose. Inhibitory substances were produced by heating glucose with sodium hydroxide or with hydrochloric acid, and the inhibitory effects were counteracted by the presence of agar in the mediums. He reported that increasing concentrations of glucose ($M/80$ to $M/20$) in amino acid medium progressively decreased alpha-hemolysin titers in the absence of agar while increasing the titers if agar was present in the medium. The depressing effect of glucose reported by Gladstone[9] was not attributed to reduction in pH.

The above reported findings concerning alpha-hemolysin production are of interest in the present investigation, since it might be of great practical importance if an inhibitor of enterotoxin formation could be found. Our findings, in general, confirm the inhibiting effect of high glucose concentration on production of both alpha- and beta-hemolysins, although we have found no definite indication in our amigen or veal infusion broth mediums of inhibitors which can be adsorbed on cellophane. No indications of specific inhibitors of either type have been obtained for enterotoxin production; however, this should be reinvestigated when a better assay method is available.

Also of interest were reports in the literature of the bacteriostatic properties of quercetin. Quercetin is a highly colored, aglycone derivative of rutin (a flavone glucoside normally present in a variety of plants such as buckwheat, tobacco, garden rue, forsythia, elder flowers and violets). It has been reported to inhibit growth of staphylococci[13] in a concentration of 0.1 mg/ml and *Clostridium botulinum*[14] in concentrations as low as 20 ppm. Preliminary to the investigation of its possibilities as a toxin inhibitor, the effects of varying concentrations of quercetin* (0.010 mg/ml to 0.363 mg/ml) on the growth of staphylococci were studied in amigen medium and in veal infusion broth (with inoculums ranging from 0.2 ml to 0.2 $\times 10^{-9}$ ml of broth culture). Some decrease of growth rate occurred in concentrations of 0.010 to 0.363 mg/ml. Inhibition varied directly with the concentration of quercetin and inversely with the size of the inoculum. Inhibition was greater in veal infusion broth and was probably related to the greater solubility of quercetin in this medium than in amigen medium. Incubation of cultures for 7 to 14 days allowed maximum growth in amigen and also in veal infusion broth except in quercetin concentrations of 0.1 mg/ml or higher. Because of the questionable value of quercetin as a growth-inhibiting agent, its relatively poor solubility, and its brown color in solution, this compound was not investigated further.

13. Naghski, J., Copley, M. J. and Couch, J. F. 1947, Science, **105**: 125–126.

14. Anderson, A. A. and Berry, J. A. 1947, Science, **106**: 644–645.

SUMMARY

Three methods have been employed for producing large volumes of *Staphylococcus* enterotoxin in hydrolyzed casein mediums. These are:

1. *Shallow cultures.*—Fifty Roux bottles, each containing 200 ml of culture, are incubated at 37 C in a sealed incubator containing 20% carbon dioxide and 80% air.

2. *Deep cultures in turning bottles.*—A sealed 12-gallon bottle containing 10 L of culture under 20% carbon dioxide plus 80% air is turned continuously in a horizontal position on rollers during incubation at 37 C.

3. *Deep aerated cultures.*—One to 15 L of deep culture is aerated with either air or a 20% carbon dioxide plus 80% oxygen gas mixture during incubation at 37 C.

* Received from Dr. James F. Couch.

SOME PHYSIOLOGICAL PROPERTIES OF NONPATHOGENIC MYCOBACTERIA

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The importance of nonpathogenic members of the genus *Mycobacterium* lies in the fact that they may be present in materials under examination which are suspected of containing tubercle bacteria. Literature concerning saprophytes is meager. Many of the physiological reactions of saprophytes presented in the 6th edition of Bergey's Manual[1] are based largely on the findings of Gordon[2] and Gordon and Hagan.[3] However, carbohydrate utilization by many members of the genus *Mycobacterium* has been extensively studied by Merrill.[4–8] This investigator employed a synthetic medium with a single carbon source. By this means, growth and pH changes depended upon the utilization of the carbon compound.

One of the difficulties encountered in estimating growth on various substrates turbidimetrically lies in the fact that these organisms grow only as a pellicle and are unable to produce an even turbidity throughout a liquid medium. Dubos[9,10] and Davis and Dubos,[11,12] however, showed that dispersed growth of the human strain of tubercle bacteria could be obtained.

The purpose of this investigation was to study some of the physiological reactions of typical organisms in this group in the hope of adding to our understanding of their biology.

MATERIALS AND METHODS

Cultures.—The results presented here are on 13 organisms selected from a large group, many of which had reactions similar to those herein reported. The species names and sources of the cultures are listed in table 1. Stock cultures were maintained on 5% glycerol agar slants. All cultures were incubated at 35 C for 7 days unless otherwise stated.

Carbon compounds.—Ten percent solutions of the following carbon compounds were prepared: sodium citrate, sodium acetate, sodium lactate,

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1. Breed, R. S., Murray, E. G. D. and Hitchens, A. P. 1948, Bergey's Manual of Determinative Bacteriology. ed. 6, Baltimore, Williams and Wilkins Co., pp. 875–891.
2. Gordon, R. E. 1937, The classification of acid-fast bacteria. I. J. Bact. **34**: 617–630.
3. Gordon, R. E. and Hagan, W. A. 1938, The classification of acid-fast bacteria. II. J. Bact., **36**: 39–46.
4. Merrill, M. H. 1928, Factors concerned in demonstrating utilization of carbohydrates by organisms of the genus *Mycobacterium*. Proc. Soc. Exper. Biol. Med. **25**: 574–575.
5. Merrill, M. H. 1929, Studies in carbohydrate utilization by organisms of the genus *Mycobacterium*. Proc. Soc. Exper. Biol. Med. **27**: 164–166.
6. Merrill, M. H. 1930, Carbohydrate metabolism of organisms of the genus *Mycobacterium*. J. Bact. **20**: 235–286.
7. Merrill, M. H. 1931a, Studies on carbon metabolism of organisms of the genus *Mycobacterium*. II. Utilization of organic compounds in a synthetic medium. J. Bact. **21**: 361–374.
8. Merrill, M. H. 1931b, Studies on carbon metabolism of organisms of the genus *Mycobacterium*. III. End products of carbohydrate utilization as determined in synthetic media cultures. J. Bact. **21**: 375–381.
9. Dubos, R. J. 1945, Rapid and submerged growth of mycobacteria in liquid media. Proc. Soc. Exper. Biol. Med. **58**: 361–362.
10. Dubos, R. J. 1946, Effect of long chain fatty acids on bacterial growth. Proc. Soc. Exper. Biol. Med. **63**: 56–58.
11. Davis, B. D. and Dubos, R. J. 1946a, Interaction of serum albumin, free and esterified oleic acid and lipase in relation to cultivation of the tubercle bacillus. Arch. Biochem. **11**: 201–203.
12. Davis, B. D. and Dubos, R. J. 1946b, Serum albumin as a protective rather than nutritive growth factor in bacteriological media. Federation Proc. **5**: 246.

sodium formate, ammonium tartrate, glucose, sucrose, arabinose, glycerol and mannitol. Ten ml of the 10% solution were added aseptically to give a final concentration of 1%. Due to the low solubility of dulcitol, sorbitol, sodium oxalate, sodium ethyl oxalacetate, and sodium succinate, final concentrations of 0.5% were used. Sodium fumarate was used in a final concentration of 0.025%. All of the carbon compounds were sterilized by Seitz filtration with the exception of glycerol, which was autoclaved.

Utilization of various carbon compounds.—Because of discrepancies in the literature regarding the utilization of various carbon compounds, it was considered necessary to reinvestigate the problem, using a wide variety of carbohydrates, alcohols, and organic acids and in particular compounds not previously tested. The synthetic medium used by Merrill,[8] which contained the carbon compound under investigation as the sole source of carbon, was employed in these studies. Growth and a reaction change in the medium indicated utilization. The medium had the following composition: 5 g sodium chloride, 5 g ammonium sulfate, 0.005 g potassium diphosphate, 0.4 g potassium monophosphate, 0.01 g phenol red, carbon source as indicated above; and it was made to 1000 ml with distilled water. Each constituent was dissolved in distilled water, autoclaved individually and added in such quantities to make a medium containing the desired concentrations.

The carbon source was added to the salts solution and mixed thoroughly. Transfers were made from stock cultures to 5 ml of medium and incubated for a period of 14 days. Determinations of growth by pellicle formation and color changes in indicator were noted. Thirteen carbon compounds were tested in this manner.

Growth in various carbon compounds in the presence of Tween 80 as a dispersing agent.—In order to determine the relative availability of the various carbon sources, it was thought advisable to measure growth response turbidimetrically. Since the use of the turbidimetric method for growth measurement requires a suspension of the organisms, Tween 80 was incorporated in the medium as a dispersing agent. The medium employed was identical with that above except that the indicator was omitted and 0.5% Tween 80, 0.2% bovine serum albumin, and 0.01% sodium fluoride were added.[9–12] None of the cultures used in this study were able to grow in the presence of these three substances combined.

Fifty ml of medium in 125 ml Erlenmeyer flasks were inoculated with 0.1 ml of a fluid culture of the organism grown in the particular carbon source used in the experiment. Twelve carbon compounds were tested in this manner. The flasks were incubated at 35 C for 7 days. Turbidity measurements were made by means of a Klett-Summerson photoelectric colorimeter (blue filter, 420 mμ). Changes in pH were determined with a glass electrode.

Effects of vitamins and liver extract.—The growth stimulating effects of 12 vitamins and a liver extract incorporated in Merrill's synthetic medium were determined separately using *Mycobacterium phlei* 354 as the test organism. The following vitamin concentrations were employed: 1 μg/ml each of p-aminobenzoic acid, thiamine hydrochloride, pyridoxine hydrochloride, choline chloride, riboflavin, inositol, calcium D-pantothenate, and niacin, and 0.1 μg/ml each of biotin, glutathione, pteroylglutamic acid and B_{12}. A 1% solution of liver extract (fraction L, Wilson) was prepared by dissolving the extract in distilled water and neutralizing to pH 7.0 with sodium hydroxide. After sterilization in the autoclave, it was added to the medium in final concentration of 0.05%.

Control mediums were prepared using the most favorable and a less favorable carbon source, glycerol and sodium citrate, respectively. The effects produced by vitamins and liver extract were determined by turbidity and pH readings after one week as compared to control flasks containing no such additions.

RESULTS

Utilization of various carbon compounds.—It can be seen in table 1 that the carbon compounds here employed could be utilized by most of the test organisms. However, the varied utilization of several of the carbon sources may be differential value. It is of interest to note that with the possible exception of citrate and oxalate, acid formation is confined chiefly to the alcohols.

Growth in various carbon compounds in the presence of Tween 80 as a dispersing agent.—Tween 80 was especially effective in producing dispersed growth of the organisms. It should be emphasized that the cultures studied did not grow in Tween 80 alone. Results of total growth on various carbon compounds are presented in table 2. Most of the cultures produced greater turbidities when glyc-

TABLE 1.—*Reaction changes of phenol red indicator in synthetic mediums containing the carbon compounds indicated as the sole source of carbon.*

Organism	Glycero	Mannitol	Dulcitol	Sorbitol	Sodium acetate	Sodium citrate	Sodium formate	Sodium lactate	Sodium oxalate	Ammonium tartrate	Sodium ethyl oxalacetate	Sodium fumarate	Sodium succinate
M. leprae 69*	A	A	A	A	B	—	B	B	B	B	B	B	B
M. smegmatis 101*	A	A	A	—	B	—	B	B	B	B	B	—	B
M. ranae 110*	A	A	A	A	B	A	B	B	B	B	B	B	B
M. tuberculosis var. *hominis* 279*	A	A	A	A	B	A	B	B	B	B	B	B	B
M. avium 7992*	A	A	A	A	B	A	B	B	B	B	B	—	B
Unnamed 13†	A	A	A	—	B	A	B	B	A	A	A	B	B
Unnamed 112†	A	A	—	—	B	—	B	B	A	—	—	B	B
M. thamnopheos 4445*	A	A	—	—	B	B	B	B	—	—	—	B	B
M. lacticola 9626*	A	A	—	—	B	—	B	B	—	—	—	B	B
M. phlei 8a†	A	A	—	A	B	—	B	B	A	—	—	B	B
M. phlei 39‡	A	A	—	A	B	—	B	B	A	—	—	B	B
M. phlei 354*	A	A	—	A	B	—	B	B	A	—	—	B	B
M. phlei 10142*	A	A	—	A	B	—	B	B	A	—	—	B	B

A = acid reaction; B = alkaline reaction; — = no growth.
* Obtained from the American Type Culture Collection, Washington, D. C.
† Obtained from Dr. R. E. Gordon, American Type Culture Collection, Washington, D. C.
‡ Obtained from Dr. D. Wingate, Duke University, Durham, N. C.

TABLE 2.—*Growth (% light transmission) and changes in pH obtained in synthetic mediums which contained Tween 80 and the carbon compounds indicated.**

Carbon Source	*M. phlei* 8a	Unnamed 13	*M. phlei* 39	*M. leprae* 69	*M. smegmatis* 101	*M. ranae* 110	Unnamed 112	*M. tuberculosis* var. *hominis* 279	*M. phlei* 354	*M. avium* 7992	*M. lacticola* 9626	*M. phlei* 10142
	pH and % light transmission											
Glycerol	6.3 23	6.4 28	6.7 92	6.2 26	6.2 21	6.4 28	6.4 38	6.6 33	6.2 20	6.3 24	6.4 50	6.3 25
Mannitol	6.2 35	6.1 30	6.3 29	6.3 30	6.2 29	6.2 26	6.3 53	6.3 32	6.2 28	6.3 25	6.3 30	6.3 29
Dulcitol	6.0 39	6.0 43	5.8 45	6.0 37	6.0 32	6.0 32	6.5 58	6.0 38	6.0 36	6.0 35	6.3 40	6.0 38
Sorbitol	6.5 39	6.5 45	6.5 31	6.4 37	6.4 29	6.7 29	6.7 55	6.5 35	6.4 31	6.5 30	6.5 37	6.4 32
Sodium lactate	6.5 50	6.4 41	6.6 35	6.9 52	6.9 34	6.9 37	6.7 97	6.8 57	6.5 49	6.7 44	6.9 81	6.5 49
Sodium acetate	6.9 97	7.1 65	6.8 75	6.9 47	6.8 43	6.8 45	6.9 100	7.0 42	6.8 94	6.8 44	6.8 73	6.8 59
Ammonium tartrate	6.5 41	6.4 35	6.4 29	6.4 30	6.5 27	6.4 27	6.6 73	6.4 33	6.4 32	6.5 31	6.5 35	6.4 34
Sodium oxalate	6.7 61	6.5 37	6.6 34	6.5 43	6.7 36	6.6 35	6.8 97	6.6 45	6.5 34	6.5 36	6.7 81	6.5 38
Sodium citrate	6.9 100	6.7 54	6.7 47	6.9 100	6.8 96	6.8 73	6.9 100	6.9 100	6.8 93	6.8 59	6.9 100	6.8 98
Arabinose	6.5 46	6.5 37	6.4 28	6.4 36	6.3 27	6.4 28	6.6 54	6.3 32	6.4 34	6.3 28	6.6 38	6.3 34
Glucose	6.5 41	6.6 47	6.5 30	6.5 36	6.5 33	6.5 32	6.7 60	6.7 36	6.5 35	6.5 37	6.7 46	6.5 36
Sucrose	6.4 40	6.5 40	6.5 31	6.5 41	6.4 32	6.4 29	6.6 57	6.5 35	6.4 36	6.4 33	6.6 39	6.5 38

* Control mediums were all pH 6.9 ± 0.1 and gave 100% light transmission. *M. thamnopheos* 4445 was not available for this experiment.

erol was the sole source of carbon. In all instances where the greatest turbidity was produced with glycerol, mannitol was second. Mannitol gave the highest turbidities with *Mycobacterium ranae* 110, *Mycobacterium tuberculosis* var. *hominis* 279, and *Mycobacterium lacticola* 9626.

The pH changes with respect to the carbon sources employed are presented in table 2. It can be noted that in nearly every case, a decrease in pH occurred. Comparison with table 1 shows that whereas alkaline reactions were produced in some indicator studies, acid reactions were produced by nearly every culture when Tween 80 was present. It should also be noted that with a progressive increase in growth there was a subsequent progressive decrease in pH.

Effect of vitamins and liver extract.—The results of turbidimetric measurements when these substances were included in the medium showed that the vitamins were without effect and that liver extract was only slightly stimulatory.

DISCUSSION

A survey of the literature revealed discrepancies concerning the utilization of various carbon sources, which may be attributed to the interpretation of results obtained by earlier investigators. In instances where a synthetic medium was not employed, an acid reaction was indicative of utilization of the carbon compounds. On the other hand, an alkaline reaction indicated that some other constituent of the medium, e.g., peptone, was being utilized. Weinzirl and Knapton[13] reported that these organisms apparently were not able to utilize glucose, mannitol, and lactose because progressive alkaline reactions were produced.

The results recorded in table 1 were obtained when a single carbon source was incorporated in a synthetic medium containing phenol red as an indicator. The carbon compound constituted the only source of carbon and utilization was demonstrated by the ability of the organisms to grow regardless of the final reaction produced in the medium. Merrill[6,8] demonstrated quite conclusively that these organisms, if able to attack a carbohydrate molecule, oxidized it completely to carbon dioxide and water without the accumulation of demonstrable amounts of intermediate products in the medium. It is interesting to note that acid reactions were produced in polyhydric alcohols and alkaline reactions predominately in the salts of organic acids.

A comparison of the results of this study with those of Merrill reveals that acid reactions were produced when the cultures were grown in polyhydric alcohols. However, whereas Merrill found no acid produced from mannitol, all cultures used in this investigation produced acid reactions regardless of the presence or absence of Tween 80. There is no way of comparing the submerged growth obtained with Tween 80 and the previous experiments of Merrill with sealed cultures.

Turbidimetric measurements of growth response to the various carbon compounds was accomplished by incorporating Tween 80 in the medium as a dispersing agent. These studies demonstrated that glycerol provides the greatest turbidity in nearly all cases. This is contrary to the findings of Plotho,[14] who

13. Weinzirl, J. and Knapton, F. 1927, The biology of the tubercle bacillus. I. Hydrogen ion concentration produced by some members of the genus *Mycobacterium*. Am. Rev. Tuberc. **15**: 380–388.

14. Plotho, O. V. 1942, Untersuchungen zur Morphologie und Biologie des Mykobakterium des Bodens. Arch f. Mikrobiol. **13**: 93–137.

reported that except for *M. lecticola*, glucose was more suitable for growth of mycobacteria. Mannitol gave turbidities almost equivalent to glycerol. When Tween 80 was present in the medium, acid reactions were produced with nearly all of the carbon sources, while in the absence of Tween 80, alkaline reactions occurred predominately in salts of organic acids.

Though the literature contains reports concerning Tween 80 as a dispersing and stimulatory agent for tubercle bacteria, no report has come to our attention concerning the use of this agent in studies with saprophytic members of the genus.

Since 12 vitamins had no effect on the growth of *M. phlei* 354 whereas liver extract was only slightly stimulatory, one might suspect that the organism was able to synthesize all necessary elements for growth on simple mediums.

SUMMARY

The physiological reactions of a collection of saprophytic, acid-fast organisms were studied in an endeavor to further characterize them. The organisms of the genus *Mycobacterium* included in this study were able to obtain their carbon and energy supply from some carbohydrates, polyhydric alcohols, and salts of organic acids when these compounds were present in a synthetic medium and constituted the sole source of carbon. Utilization was indicated by growth and change in pH. Utilization of the carbon compounds by a number of the organisms is of sufficiently varied nature to be of value as a means of differentiating some of the species.

Dispersed growth of the organisms was obtained by adding Tween 80 to the medium. Turbidimetric measurements demonstrated that glycerol afforded the best carbon source for growth in most cases. There was an inverse relationship in the amount of growth and pH observed.

The addition of vitamins or liver extract to the medium did not provide any pronounced effect on the amount of growth.

STUDIES OF THE EFFECT OF ANTIBIOTICS ON INFECTIONS WITH THE MOUSE PINWORM, *ASPICULURIS TETRAPTERA*

I. THE ACTION OF TERRAMYCIN HYDROCHLORIDE

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We have been interested in using the mouse pinworm, *Aspiculuris tetraptera*, for evaluating new drugs which may be active against the human pinworm, *Enterobius vermicularis*. *A. tetraptera* may be of value in such therapeutic tests, since it is a related nematode and parasitizes the large intestine of its host. In this first report are presented our results with terramycin.

METHODS AND MATERIALS

For these studies a strain of mice free of helminths has been reared. The colony was started with the Carworth Farms CF 1 Albino strain, and the mice were rendered helminth free by vigorous treatment with Phenothiazine and castor oil. The animals were reared in an isolated room, and special precautions were taken in regard to all food, animal pans, and sawdust, which were previously sterilized.

To produce experimental infections with *Aspiculuris*, stock infected mice were autopsied and gravid female worms were secured. These worms were broken up in distilled water and the eggs were incubated at 27 C for 7 days, the optimum time for obtaining the maximum number of infective embryonated eggs. This optimum embryonation time confirms a previous report of Philpot.[1]

The dosage of eggs was determined by a dilution counting method in which only embryonated eggs were counted. All mice were given 300 embryonated eggs, administered into the esophagus with a blunt tipped needle and tuberculin syringe. The mice used were between 25 and 30 days of age at the start of each experiment, and in each experiment 15 mice were used as controls and 15 for chemotherapeutic testing. Treated animals were given a single daily dose of terramycin at a rate of 500 mg/kg of body weight by stomach tube. The mice were autopsied at definite intervals after infection and the entire large intestine was examined microscopically and the worms counted.

In every experiment except the first, the lengths of worms were measured according to the procedure of Rappaport and Wells.[2] Wherever possible 10 worms were measured from each of 5 animals in a group. In all instances data were considered to be statistically significant when the difference in means divided by standard error of the difference in means is at least 2.5.

EXPERIMENTAL

Experiments 1a and 1b.—These series are identical except that they were carried out at different times. The experiments were designed to study the effect of terramycin on immature larval forms. The treated mice received daily doses of 500 mg/kg of terramycin over a period of 16 days beginning two days prior to infection with *Aspiculuris*. The day after receiving the last dose of

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1. Philpot, F. 1924, Notes on the Eggs and Early Development of Some Species of Oxyuridae. J. Helminthol. **21**: 239.

2. Rappaport, I. and Wells, H. S. 1951, Studies in Trichinosis. I. Immunity to Reinfection in Mice Following a Single Light Infection. J. Infect. Dis. **88**: 248–253.

TABLE 1.—*Effect of terramycin hydrochloride on* Aspiculuris tetraptera *worm burden in mice. All animals received 300 eggs.*

Exp. no.	Treated animals				Control animals		
	No. of mice	1st day of treatment	Total days of treatment (500 mg/kgm daily)	Average worm yield	No. of mice	Average worm yield	t*
1a	15	2 days prior to infection	16	4.2	15	74.2	3.7†
1b	15	2 days prior to infection	16	12.1	15	97.3	6.4†
2	15	7 days after infection	7	115.7	15	97.3	0.87
3	15	7 days after infection	14	13.0	15	65.0	6.1†
4	15	14 days after infection	14	37.3	15	56.7	1.21

* Differences in means divided by the standard error of the difference in means.
† Indicates significant difference between treated and control animals.

TABLE 2.—*The effect of terramycin hydrochloride on lengths of* Aspiculuris tetraptera.

Exp. no.	Treated animals		Control animals		
	No. of worms measured	Average worm length	No. of worms measured	Average worm length	t*
1b‡	41	0.84 mm	50	*1.37 mm	14.0†
2	50	1.32 mm	50	1.37 mm	2.0
3	43	1.48 mm	50	2.92 mm	17.1†
4	34	3.28 mm	49	3.73 mm	4.2†

In each experiment worms from 5 animals were measured, and wherever possible, 10 worms were measured from each animal.
* Difference in means divided by the standard error of the difference in means.
† Indicates significant difference between treated and control animals.
‡ Measurements of both males and females.

terramycin, the mice were autopsied and the worms were counted (i.e. 14 days after infection). The results of worm yields of these series are found in table 1. In experiment 1a the average worm yield in control mice was 74.2 and in treated mice, 4.2 In experiment 1b the average for control mice was 97.3 worms and for treated mice 12.1. In both series the difference in worm yields between treated and control mice proved to be statistically highly significant. Worm measurements were made only in experiment 1b, the results of which are found in table 2. The average length of worms in control animals was 1.37 mm and for the terramycin treated animals, 0.84 mm, a highly significant difference.

Experiment 2.—In this experiment treatment was begun 7 days after infection and continued for 7 days. The controls were the same as in experiment 1b. The average worm yield in these treated animals was 115.7, which on statistical analysis proved to be not significantly different from the control yield of 97.3. The average worm length was 1.32 mm, again not significantly different from the average control length of 1.37 mm.

Experiment 3.—This experiment was designed to determine whether terramycin was active only after prolonged treatment or exerted its effect only on very young larvae. Here, terramycin was administered 7 days after infection and continued for 14 days. Mice were autopsied 21 days after infection. The results may be seen in tables 1 and 2. The average worm yield in treated animals was 13.0 and in control animals 65.0, a significant difference. Worm measurements were made only on females, since very few male worms were recovered in treated animals. In treated animals the average female worm length was 1.48 mm and in controls 2.92 mm, a significant difference.

Experiment 4.—The object of this experiment was to determine the action

of the antibiotic on maturing worms already sexually differentiated and on the fecundity of the worms. By the 28th day of infection female worms are gravid and eggs are found in the mouse feces. In this series treatment was begun after 14 days of infection, continued for 14 days thereafter, and the mice were then autopsied. The results are found in tables 1 and 2. The average worm yield of the controls was 56.7 and the treated was 37.3, a difference which is not statistically significant. In control animals the average length of female worms was 3.73 mm and in treated animals it was 3.28 mm, a significant difference.

In comparing the fecundity of the females in control and treated animals, it was noted that all controls contained gravid females. Of the 15 treated animals, 7 contained gravid females whose uteri were found to contain fewer eggs than the controls; in 2 animals the worms contained very few eggs; in 3 animals the uteri were entirely empty; and in 3 no females were recovered.

DISCUSSION

From the above data it is evident that terramycin affects the course of the infection and the development of *Aspiculuris tetraptera* in several ways: it decreases the worm burden, reduces the size of retained worms, and apparently, during the course of treatment, diminishes egg production.

Treatment for 7 days was insufficient to produce these effects at a stage in the life cycle when the worms appeared to be most susceptible to prolonged treatment. In those experiments where prolonged treatment was employed the best results were obtained when mice were treated for a total of 16 days, beginning two days prior to infection. The poorest therapeutic effects as regards reduction in worm burden were obtained in animals harboring mature worms. It appears that in animals treated from the first to the 14th day of infection, the sizes of worms are approximately those of normal 7-day-old worms. Worms after the 7th day of infection were found to be on the average 0.76 mm in length. In those animals treated from the 7th through the 21st day the average worm size is that of control 14-day worms. However, in those animals treated from the 14th through the 28th day of infection, size reduction although significant statistically, is not of the magnitude of the reduction in size of younger forms.

We do not as yet know the mechnism of action of terramycin on *Aspiculuris tetraptera*, nor can we explain the greater therapeutic action against younger forms. We are now attempting to determine whether the action of terramycin is directly on the worm or whether it may affect the nutrition of the worm through alteration of the intestinal flora of the host. The action of other antibiotics on both the worm and the intestinal flora of the host are being studied in an attempt to shed further light on this problem. Clinical investigations are in progress to determine whether terramycin exerts a similar therapeutic effect on human infection with *Enterobius vermicularis*.

SUMMARY

1. A laboratory method for investigating the action of drugs against the mouse pinworm is presented.

2. Terramycin hydrochloride is effective in reducing the mouse infection with *Aspiculuris tetraptera* as manifested by a reduction in worm burden, reduction in size of retained worms, and diminished egg production.

3. Terramycin is especially effective against immature forms.

4. For the above effects it was necessary to continue treatment for 14 days.

PROPHYLAXIS OF EXPERIMENTAL DIPHTHERIA WITH PENICILLIN

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In 1929, Fleming,[1] after his observations of the anti-bacterial action of penicillin, reported that a slightly purified extract of a broth culture was fairly active against the etiological agent of diphtheria, *Corynebacterium diphtheriae*. With the development of purified penicillin, in vivo tests were proposed using penicillin as a prophylactic agent in diphtheritic infections in laboratory animals. The guinea pig was employed because of the relative refractiveness of the mouse to diphtheritic infection.[2–8]

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1. Fleming, A. 1929, On the Antibacterial Action of Cultures of a Penicillin with Special Reference to Their Use in the Isolation of *B. influenza*, Brit. J. Exper. Path. **10**: 226–236.
2. Coca, A. F., Russel, E. F. and Baughman, W. H. 1921, The Reaction of the Rat to Diphtheria Toxin. J. Immunol. **6**: 387–388.
3. Glenny, A. T. and Allen, K. 1932, The Action of Diphtheria Toxin on Mice. J. Hyg. **21**: 96–99.
4. Goodman, H. M. 1941, Cited by Seligman, E. and Jungeblut, C. W. Experimental Diphtheria in the Albino Rat. J. Immunol. **40**: 119–126.
5. Loeffler, F. 1884, Untersuchungen über die Bedeutung der Mikroorganisemen. Mitth. a.d.k. Gsndhtsamte, **2**: 421–499.
6. Loeffler, F. 1890, Bemerkungen zu der Arbeit von Prof. E. Klein "Zur Aetiologen der Diphtherie," Zentralbl. f. Bakt. **7**: 528–529.
7. Roux, E. and Yersin, A. 1888, Contribution à L'étude de la Diphtérie. Ann. Inst. Pasteur, **2**: 629–661.
8. Wernicke, G. 1946, Cited by Wilson, G. G. and Miles, A. A., Topley and Wilson's Principles of Bacteriology and Immunity, ed. 3, Baltimore, William and Wilkins Co.

Ercoli,[9] in 1945, reported that a dose of penicillin delayed the death of guinea pigs infected with cultures of *C. diphtheriae*; also, that penicillin exerted a marked activity against systemic, mainly bacillary, infections in mice. In the same year, Young and Mood[10] observed that penicillin was very active in extremely small amounts in preventing diphtheritic infections and fatal toxemia in guinea pigs.

Recently, prolonged therapeutic blood levels have been obtained following a single injection of penicillin-procaine.[11,12] Inhibitory levels have been maintained for as long as 4 or 5 days. The possibility of using penicillin-procaine as a prophylactic agent against diphtheritic infections suggests itself. Our results in experimental animals indicate that protection may be obtained with its use.

MATERIALS AND METHODS

The culture of *C. diphtheriae*† #49680,

9. Ercoli, N., Lewis, M. N. and Moench, L. J. 1945, The Antibacterial Activity of Penicillin in Experimental Infections of Mice with *C. diphtheriae*. J. Pharmacol. & Exper. Therap. **84**: 120–127.
10. Young, R. M. and Mood, G. McF. 1945, Effect of Penicillin on Infection of Guinea Pigs with *C. diphtheriae*. J. Bact. **50**: 205–212.
11. Herrell, W. E. 1947, Procaine Penicillin G (Duracillin). Proc. Staff Meet. Mayo Clinic, **22**: 567–570.
12. Sullivan, N. P., Symmes, A. T., Niller, H. C., Rhodehamel, H. W. 1948, A New Penicillin for Prolonged Blood Levels, Science, **107**: 169–170.

† Obtained through the courtesy of Miss Marion Coleman, New York State Department of Health, Albany, New York.

gravis strain, was found to be pathogenic for mice. The organisms were grown on Loeffler's serum agar slants and transferred to fresh slants every month. The slants were incubated at 37 C for 48 hours after which they were stored at 4 C. Transfers were obtained and grown on Loeffler's serum agar in 8 ounce, screw-capped bottles. Before autoclaving, the bottles were placed on their sides so a suitable area for growth was available. After inoculation, the bottles were incubated at 37 C for 48 hours.

The organisms were suspended in sterile distilled water, centrifuged at approximately 283 g for 15 minutes, resuspended in sterile distilled water to a matched turbidity comparable to a #10 tube of a McFarland nephelometer series.[13] Plate counts were made, using tryptose phosphate agar, to determine the number of viable organisms present.

The number of organisms necessary to produce 100% mortality in Harlan strain, albino male mice, weighing 15 to 20 g, was determined by injecting mice intraperitoneally with various dilutions of the suspension. The LD_{100} dose that killed the mice in 3 to 5 days was selected as the infective dose and contained 0.5 to 2.25 billion viable organisms per 0.5 ml. Fresh suspensions were prepared for each series of experiments performed.

In vitro studies

Aqueous penicillin G sodium salt, containing 5000 units per ml, was the antibacterial agent used. Tryptose phosphate broth was employed to demonstrate growth inhibition of a 24-hour culture of *C. diphtheriae* incubated at 37 C. Two controls were prepared; one contained no penicillin, the other also contained no penicillin but was heated at 80 C for 20 minutes. Inoculated tubes were incubated at 37 C and the readings were taken after 24 and 48 hours (table 1). After the last reading, 0.1 ml of penicillinase,‡ containing 400 units, was added, and the tubes were reincubated at 37 C for 48 hours.

TABLE 1.—*In vitro effects of aqueous penicillin G sodium salt on the growth of* C. diphtheriae *at 37 C for 48 hours.*

Tube no.	Penicillin		Broth ml	Broth culture ml	Growth	
	Units	ml			without penicillinase	with
1	50.00	0.1	4.4	0.5	−*	+†
2	10.00	0.1	4.4	0.5	−	+
3	5.00	0.1	4.4	0.5	−	+
4	1.00	0.1	4.4	0.5	+	+
5	0.50	0.1	4.4	0.5	+	+
6	0.10	0.1	4.4	0.5	+	+
7	0.05	0.1	4.4	0.5	+	+
8	0.03	0.1	4.4	0.5	+	+
9	0.01	0.1	4.4	0.5	+	+
10	0.00	0.0	4.5	0.5	+	+
11‡	0.00	0.0	4.5	0.5	−	−

* No growth is indicated by −; growth is indicated by +.
† Penicillinase was added at 48 hours.
‡ Control was heated at 80 C for 20 minutes.

In vivo studies

Penicillin G procaine, aqueous combined with aqueous potassium penicillin, was used to determine the possible prophylactic value of the penicillin. Intraperitoneal injections of 300 and 150 units of penicillin were made in all groups of mice, except in group C, where the injection was made intramuscularly in the inner surface of the left hind leg to determine the prophylactic effect of systemic injections of penicillin. (See table 2.) The dosage of penicillin given was calculated on the basis of the body weight of a 17.5 g mouse to a 70 kg man. All mice were observed over a holding period of 20 days.

The time lapse between injections of the lethal dose and penicillin was varied

13. Kabat, E. A. and Mayer, M. M. 1948, Experimental Immunochemistry, ed. 1, Springfield, Illinois, Charles C Thomas.

‡ Supplied by Schenley Laboratories, Lawrenceburg, Indiana.

because observations had been made upon the clostridia, indicating that elapsed time plays a very important role in protection.[14]

In table 3, multiple injections of aqueous penicillin G procaine were used, with a time lapse between injections of the lethal dose and penicillin, to determine whether or not multiple injections would pose the possibility of a greater degree of protection to infected groups of animals. The second injection of penicillin was given 48 hours after the initial injection.

Penicillin G procaine in sesame oil with 2% aluminum monostearate, containing 300,000 units per ml, was used in a similar manner to test the possible prophylactic value of slowly absorbable penicillin resulting in a longer period of therapeutic activity (table 4). Injections were carried out as before.

Since penicillin does not affect the toxin produced but affects the organisms producing the toxin, antitoxin experiments were proposed for comparative studies (table 5).

TABLE 2.—*Action of aqueous penicillin-procaine G* on mice infected with diphtheria. Series I–IV.*

Group	Penicillin		Total animals	Mortality			Time to death	
	Units	Hours before		no.	%	*P* value†	mean days	*P* value‡
A	0	0	40	40	100.0		3.8	
B	150	0	40	23	57.5	<0.01	8.8	<0.01
C	150§	0	40	23	57.5	<0.01	9.5	<0.01
D	300	0	40	22	55.0	<0.01	7.6	<0.01
E	150	3	40	33	82.5	<0.02	6.0	>0.05
F	150	6	40	38	95.0	>0.05	5.9	>0.05
G	150	24	40	35	87.5	>0.05	4.4	>0.05

* Penicillin G procaine, aqueous, in a ratio of 3:1 of aqueous potassium penicillin.
† *P* values were taken from a Chi-square 4-fold table including Yates correction; all values less than 0.05 are significant in comparison with group A.
‡ *P* values were taken from Fisher's *t* table; all values less than 0.05 are significant in comparison with group A.
§ Intramuscular injection of penicillin in the inner surface of the left hind leg.

TABLE 3.—*Action of multiple injections of aqueous penicillin-procaine G* on mice infected with diphtheria. Series V–VI.*

Group	Penicillin		Total animals	Mortality			Time to death	
	Units	Hours before		no.	%	*P* value†	mean days	*P* value‡
A	0	0 48	20	20	100.0		4.2	
B	150	0 48	20	2	10.0	<0.01	15.5	<0.01
C	150§	0 48	20	1	5.0	<0.01	11.0	<0.01
D	300	0 48	20	1	5.0	<0.01	7.0	<0.01
E	150	3 51	20	6	30.0	<0.01	7.8	<0.05
F	150	6 54	20	4	20.0	<0.01	14.8	<0.01
G	150	24 72	20	12	60.0	<0.01	7.9	>0.05

* Penicillin G procaine, aqueous, in a ratio of 3:1 of aqueous potassium penicillin.
† *P* values were taken from a Chi-square 4-fold table including Yates correction; all values less than 0.05 are significant in comparison with group A.
‡ *P* values were taken from Fisher's *t* table; all values less than 0.05 are significant in comparison with group A.
§ Intramuscular injection of penicillin in the inner surface of the left hind leg.

Diphtheria antitoxin, Squibb, was supplied in a vial containing 1000 units per ml. Dilutions, in which 0.1 ml contained 2.5 units and 0.25 units, were used. The latter amount is comparable to a 1000 unit prophylactic dose in adult human beings. Injections of antitoxin were carried out as before, intraperitoneally, except in group C, where the injections were made intramuscularly in the inner surface of the left hind leg.

In table 6, antitoxin plus aqueous

14. Novak, M., Goldin, M. and Taylor, W. I. 1949, Tetanus Prophylaxis with Penicillin-Procaine G. Proc. Soc. Exper. Biol. & Med. **70**: 573–576.

TABLE 4.—*Action of penicillin-procaine G in sesame oil with 2% aluminum monostearate on mice infected with diphtheria. Series VII–VIII.*

Group	Penicillin		Total animals	Mortality			Time to death	
	Units	Hours before		no.	%	*P* value*	days mean	*P* value†
A	0	0	20	20	100.0		4.4	
B	150	0	20	5	25.0	<0.01	12.4	<0.01
C	150‡	0	20	11	55.0	<0.01	8.8	<0.01
D	300	0	20	4	20.0	<0.01	10.0	<0.01
E	150	3	20	7	35.0	<0.01	12.3	<0.01
F	150	6	20	9	45.0	<0.01	10.4	<0.01
G	150	24	20	10	50.0	<0.01	10.5	<0.01

* *P* values were taken from a Chi-square 4-fold table including Yates correction; all values less than 0.05 are significant in comparison with group A.
† *P* values were taken from Fisher's *t* table; all values less than 0.05 are significant in comparison with group A.
‡ Intramuscular injection of penicillin in the inner surface of the left hind leg.

TABLE 5.—*Action of diphtheria antitoxin on mice infected with diphtheria. Series IX–X.*

Group	Antitoxin		Total animals	Mortality			Time to death	
	Units	Hours before		no.	%	*P* value*	days mean	*P* value†
A	0.00	0	20	20	100.0		3.9	
B	0.25	0	20	17	85.0	>0.20	6.6	>0.05
C	0.25‡	0	20	14	70.0	<0.05	7.4	>0.05
D	2.50	0	20	7	35.0	<0.01	8.0	<0.05
E	0.25	3	20	16	80.0	>0.10	8.6	<0.05
F	0.25	6	20	12	60.0	<0.01	6.6	>0.05
G	0.25	24	20	15	75.0	>0.05	7.5	>0.05

* *P* values were taken from a Chi-square 4-fold table including Yates correction; all values less than 0.05 are significant in comparison with group A.
† *P* values were taken from Fisher's *t* table; all values less than 0.05 are significant in comparison with group A.
‡ Intramuscular injection of antitoxin in the inner surface of the left hind leg.

TABLE 6—*Action of diphtheria antitoxin and aqueous penicillin-procaine G* on mice infected with diphtheria. Series XI–XII.*

Group	Units		Hours before	Total animals	Mortality			Time to death	
	Penicillin	Antitoxin			no.	%	*P* value†	days mean	*P* value‡
A	0	0.00	0	20	20	100.0		4.3	
B	150	0.25	0	20	4	20.0	<0.01	9.3	<0.01
C	150§	0.25§	0	20	7	35.0	<0.01	10.6	<0.01
D	300	2.50	0	20	1	5.0	<0.01	12.0	<0.01
E	150	0.25	3	20	9	45.0	<0.01	11.4	<0.01
F	150	0.25	6	20	6	30.0	<0.01	8.3	<0.01
G	150	0.25	24	20	10	50.0	<0.01	11.3	<0.01

* Penicillin G procaine, aqueous, in a ratio of 3:1 of aqueous potassium penicillin.
† *P* values were taken from a Chi-square 4-fold table including Yates correction; all values less than 0.05 are significant in comparison with group A.
‡ *P* values were taken from Fisher's *t* table; all values less than 0.05 are significant in comparison with group A.
§ Intramuscular injection of antitoxin and penicillin in the inner surface of the left hind leg.

penicillin G procaine were used to determine the protection obtained by using two prophylactic agents, since antitoxin will neutralize the toxin produced while penicillin is effective against the organism, *C. diphtheriae*, as shown by the in vitro studies. The same procedure was carried out as employed in the preceding experiments using the same amounts of penicillin and antitoxin.

Statistical analysis

A statistical analysis of the experimental results involving the mortality and time to death of the mice receiving penicillin and the control animals receiving no penicillin was done to demonstrate the possible significance of prophylaxis of experimental diphtheria with aqueous penicillin G procaine.

The P value (probability of error

because of chance alone) of the mortality of each series as compared with the controls was obtained from a Chi-square test of independence 4-fold table, including Yates correction. Values less than 0.05 are significant. The analysis of the variable time to death in days in each group, as compared to group A, included the mean time to death and the P value from Fisher's t table, with values less than 0.05 being significant.[15]

DISCUSSION

Relative to the in vitro experiment, growth of the culture of *C. diphtheriae* #49680 was bacteriostatically inhibited by concentrations of aqueous penicillin G sodium salt containing 5 units per 0.1 ml. The data tabulated in table 2 represent the results in mice infected with a virulent culture of *C. diphtheriae* with various dosages of aqueous penicillin G procaine injected intraperitoneally, intramuscularly in group C, at various time intervals. Administration of the penicillin within three hours following the initial injection of the diphtheria organisms gave a statistically significant reduction in mortality as compared with the control animals. The lowest mortalities resulted when 150 and 300 units of aqueous penicillin G procaine were injected immediately, groups B, C, and D indicating the necessity of immediate administration of the penicillin. These groups having low mortalities also had a time lapse before death occurred, indicating that in comparison to the time lapse in the control animals, the number of organisms capable of producing a fatal dose of toxin was reduced, and that only where organisms survived, multiplied and produced a lethal dose of toxin, did death result.

The indication in group C was that systemic penicillin was effective in reaching the focus of infection. Multiple injections (table 3) indicated a lower mortality can be obtained than if one used a single injection of the penicillin (table 2).

The action of aqueous penicillin G procaine in sesame oil (table 4) in mice infected with diphtheria indicated a reduction in mortality and prolongation of life of statistical importance. Systemic penicillin, group C, was not effective probably because of the relatively slow rate of absorption of the penicillin, which apparently allowed a lethal dose of toxin to be produced before the organisms were bacteriostatically inhibited.

The factors which apply to effective prophylactic use of diphtheria antitoxin in diphtheria were noted to be similar to those which regulate the effective prophylactic use of aqueous penicillin (table 5). The effectiveness of the use of either agent would be determined by the extent of toxin injury to specific tissue cells.

Table 6 indicated the prophylactic action of diphtheria antitoxin and aqueous penicillin G procaine in mice infected with diphtheria. The greatest reduction in mortality was obtained in group D, whereas groups E, F, and G showed a higher mortality rate, indicating the necessity of prompt use of the prophylactic agents.

CONCLUSIONS

Results of the in vitro studies indicated that *Corynebacterium diphtheriae* #49680 was less susceptible to the action of aqueous penicillin G sodium salt than are many strains of this species but was bacteriostatically inhibited by 5 units of the penicillin per 0.1 ml.

In vivo results showed that diphtheria antitoxin and penicillin G procaine, aqueous or in sesame oil with 2%

15. Snedecor, G. W. 1946, Statistical Methods, ed. 4, Ames, Iowa, Iowa State College Press.

aluminum monostearate, when administered systemically or locally within three hours, is prophylactically of significant value in reducing the mortality of mice infected with diphtheria. In all cases where death was not parallel to the control animals, a delayed development of symptoms resulting in death was observed. Multiple injections of aqueous penicillin G procaine afforded more protection against experimental diphtheritic infection in mice than did single injections.

Diphtheria antitoxin significantly reduced the mortality in mice, but was not of significant value in prolonging the life of the animal after infection. In using diphtheria antitoxin in conjunction with aqueous penicillin G procaine, a decided reduction of mortality was obtained which was equal to the reduction of mortality as obtained using multiple injections of aqueous penicillin G procaine. Penicillin appears to exert a prophylactic effect in experimental diphtheritic infections in mice.

FIELD TRIAL OF *SHIGELLA FLEXNERI III* VACCINE

VII. STUDIES ON ASYMPTOMATIC CARRIERS OF THE ORGANISM

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During the field trial of *Shigella flexneri III* vaccine,[1] a significant number of individuals was found whose rectal swab cultures were positive at least once for *Sh. flexneri III*.[2] None of these men had apparent symptoms of gastrointestinal illness during the shipboard studies and all denied having any diarrheal disease for a minimum of 6 months preceding initiation of the program. As a matter of convenience, such individuals were designated as asymptomatic carriers.

Due to the epidemiologic implications of the presence of asymptomatic carriers of *Sh. flexneri III* in a population containing appreciable numbers of susceptible individuals,[2] it was believed that further laboratory and clinical studies of the carrier condition were indicated. Previously collected information concerning men included in the field trial provided a logical approach to the contemplated investigations. Through cooperation of the Bureau of Medicine and Surgery and the Bureau of Naval Personnel, 24 of the group of 103 carriers earlier identified were transferred to the Naval Medical Research Institute as volunteers for the proposed observations.

During the latter part of the studies to be described, two additional men presented themselves for study; both were Hospital Corpsmen, Second Class, who were attached to the staff of the Institute. In each instance, there was a history of probable exposure to *Sh. flexneri III* infection two to four years previously. In contrast to the rest of the group, however, both men had experienced brief and irregular gastrointestinal disturbances subsequent to their initial contact, but no bacteriological studies had been made. One man had been acutely ill at the time of his initial exposure but the other had not.

It was hoped that the program planned would provide information regarding intermittency in shedding of the organisms, the focus of infection, and a suitable procedure for effectively

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1. Barnes, L. A., Bennett, I. L., Jr. and Gordon, R. S. 1949, Field trial of *Shigella flexneri III* vaccine. I. Background, scope, and organization of the program. Nav. Med. Bull. **49**: 757–774.
2. Barnes, L. A., Smith, A. B., Durant, R. C. and Dressler, H. R. 1950, Field trial of *Shigella flexneri III* vaccine. IV. Report of bacteriologic studies. Armed Forces M. J. **I**: 1285–1297.

eliminating the carrier condition. This report is concerned, therefore, with the bacteriologic studies, clinical observations, and therapeutic treatment of the carriers available.

METHODS AND MATERIALS

Histories.—The case history of each man taken during the field trial[1] was reviewed, health records were examined, and each individual was again interrogated to establish as nearly as possible the time of initiation of the infection.

Bacteriologic studies.—In view of previously observed long negative phases in culture series from carriers,[2] it was decided to examine 60 successive rectal swab specimens from each man preliminary to clinical observation and treatment in order to determine with a fair measure of confidence which ones were still carriers and to obtain additional information regarding intermittency of positive specimens. Material for culturing was taken 6 days per week until the required number had been reached.

It was necessary to recover *Sh. flexneri III* from as many specimens examined as possible by current techniques. It was desired also to obtain some evidence regarding the occurrence of antibiotic-producing bacteria of the coliform or other intestinal group. Fecal material from a rectal swab was, therefore, streaked first across S S agar and MacConkey agar in equal amounts in a divided petri dish, then across S S agar and EMB agar in a similar container, after which the cotton-tipped end of the swab was broken off aseptically and dropped into a tube containing 2.0 ml of sterile peptone solution. The divided petri dishes were manufactured commercially according to specifications furnished by the Bacteriology Division of this Institute. The tube of peptone solution was incubated for two hours at 37 C or overnight at 4 C, after which material from it was inoculated onto a blood agar plate. The blood agar culture was then incubated overnight at 37 C and examined carefully for different varieties of gram-positive organisms which, when isolated, were tested for antibiotic activity against *Sh. flexneri III*. The blood agar plates were also examined for colonies of *Sh. flexneri III* when such organisms were not found on the other primary mediums used. From the peptone solution cultures, surfaces of special mediums previously inoculated by flooding with *Sh. flexneri III* suspension were streaked for the purpose of detecting antibiotic-producing coliform organisms.

After overnight incubation at 37 C, plates of S S, MacConkey, and EMB agar which were streaked with rectal swabs the day before were examined thoroughly macroscopically and under low power magnification for the presence of colonies suspected of being *Shigella*. When present, several lactose nonfermenting colonies were transferred to TSI agar and if, after overnight incubation at 37 C, reactions typical of *Shigella* were observed a tentative serologic identification was made by mixing growth taken from the slant with monovalent *Sh. flexneri III* serum in a slide test. All suspicious cultures were purified by restreaking on MacConkey agar plates; stock cultures were made from single isolated colonies, after which biochemic and more complete serologic studies were carried out. Gram-stained smears were made and examined as necessary. All plates of primary isolation mediums were kept for several days at room temperature and examined closely for suspicious colonies not detected during the first inspections.

One strain of *Sh. flexneri III* isolated from each of 5 carriers was tested for sulfadiazine and chloramphenicol sensitivities by means of paper disks* impregnated with varying concentrations of the two agents. The same 5 cultures were tested for sulfadiazine and antibiotic sensitivities in the laboratories of the Division of Antibiotics, Food and Drug Administration, by the method promulgated by that agency.

After the preliminary series of cultures, a period of 27 days elapsed before the next procedures were undertaken; during this time, those men found still to be carriers were granted leave if they so desired. A second series of 17 rectal swab specimens was examined according to previous procedures during a period of 19 days between return of the men and the beginning of therapeutic treatment. The final series of specimens was examined during treatment and the routine continued until 60 successive observations had been made after the last dose of antibiotic.

Clinical observations.—On the 14th culture day of the second series, the men who had been retained as persistent carriers were admitted to the naval hospital for clinical observations, tests, and treatment. Included were systematic review and physical examination; blood count, sedi-

3. Philbrook, F. R., Barnes, L. A., McCann, W. J., Jr. and Harrison, R. R. 1948, Prolonged laboratory observations on clinical cases and carriers of *Shigella flexneri III* following an epidemic. Nav. Med. Bull. **48**: 405–414.

* Obtained from the Difco Laboratories, Detroit, Mich.

mentation rate, urinalysis, Kahn test, chest X-ray, cholecystogram, barium enema, biliary drainage, and proctosigmoidoscopy. In addition to the rectal swab culturing, specimens obtained during biliary drainage and sigmoidoscopy were examined for the presence of *Shigella* by inoculation onto the mediums mentioned above. A stimulant of 1.0 mg of prostigmine methylsulfate was administered intramuscularly during biliary drainage to facilitate secretion of B-bile.

One of the men had entered the hospital on the 20th culturing day of the preliminary series with symptoms and signs that led to a diagnosis of cholecystitis, acute; the attack was mild and, on a low fat diet with antispasmodic treatment, the patient became asymptomatic in a few days. During this period of hospitalization, results of complete blood count, urinalysis, serum bilirubin, van den Bergh, Kahn, serum amylase and lipase, and thymol turbidity tests were within normal limits; stools were negative for ova and parasites but ranged from negative to 2-plus for blood. Cholecystogram and a gastrointestinal X-ray series were reported as normal. Samples of B-bile cultured were negative for *Shigella*, but swab specimens of the mucosa taken through the anoscope were positive for *Sh. flexneri III*. During proctoscopic examination, a hypertrophied anal papilla was observed; this was removed and submitted for pathologic examination but, unfortunately, was not made available for bacteriologic study.

Treatment.—On the third hospital day, after completion of the clinical studies and related laboratory tests, each carrier was placed under treatment with chloramphenicol, 250 mg every three hours for a total of 15 g. Upon completion of the course of therapy, the men were discharged from the hospital, and the scheduled number of follow-up rectal swab specimens was examined bacteriologically.

RESULTS

Historical.—In a previous report,[2] a summary of data on 103 carriers was presented; portions of this concerning 24 men, together with additional information, are shown in table 1. It can be observed that the probable initial infections occurred between November 1945 and October 1948; 11 of the 24 carriers had been ill at the time of probable first exposure. It was believed that others acquired their infections, without clinical illness, during an epidemic, and that the remainder of the group became infected as a result of contact with organisms excreted by other, previously established, carriers.[2] Of interest is the fact that 22.7% of the total rectal swab specimens studied were positive for *Sh. flexneri III*, although individual recoveries ranged from 4.0% to 83.3%.

TABLE 1.—*Historical data concerning 24 men detected as* Sh. flexneri III *carriers in 1948.*

Study no.	Rate	Probable initial infection		Total previous cultures		
		Month-year	Ill	No. taken	No. positive	% positive
B 149	YN3	11-47	Yes	15	3	20.0
B 200	TE3	5-46	Yes	18	6	33.3
B 229	CS2	10-47	Yes	15	3	20.0
B 299	PN2	7-47	No	5	1	20.0
C 289	EN2	10-47	No	11	4	36.4
B 1157	SA	10-48	No	6	2	33.3
C 334	SN	11-47	No	8	1	12.5
B 423	RD3	10-47	No	15	1	6.7
B 438	SN	5-47	No	15	4	26.7
B 1008	SA	10-48	No	11	8	72.7
C 1276	SA	10-48	No	4	1	25.0
D 770	MMFA	5-48	No	27	3	11.1
E 520	SA	9-48	No	25	1	4.0
B 581	SA	2-48	Yes	16	11	68.8
C 1308	FCSN	10-47	Yes	6	5	83.3
C 624	SA	11-47	Yes	11	1	9.1
D 410	MM2	11-45	Yes	29	2	6.9
B 689	SHSN	10-47	No	14	1	7.1
B 712	SN	11-47	No	15	4	26.7
C 694	SN	11-47	Yes	12	1	8.3
B 759	SN	9-47	Yes	9	3	33.3
C 1070	SN	10-48	No	8	1	12.5
B 771	SA	11-47	Yes	3	2	66.7
B 820	SA	12-47	Yes	15	2	13.3
	Totals			313	71	22.7

Intermittency of positive cultures was described in the previous summary.[2]

Bacteriologic studies.—In the current studies, no cultures positive for *Sh. flexneri III* were obtained from 18 men during the preliminary series of 60 examinations. It was concluded, therefore, that the individuals in this group had spontaneously ceased to be carriers some time between their last previous positive culture and the beginning of the present investigations. The duration of the carrier condition under these circumstances varied, therefore, between minimum periods of 0 to 21 months and maximum times of 19 to 36 months. The bases for these estimations are given in table 2.

TABLE 2.—*Duration of* Sh. flexneri III *infection in 18 men detected as carriers in 1948.*

Study no.	Month-year of probable initial infection	Month-year of last positive culture	Months duration of infection	
			Minimum	Maximum*
B 149	11-47	11-48	12	30
B 200	5-46	2-49	21	36
B 229	10-47	2-49	16	31
B 299	7-47	8-48	13	34
C 289	10-47	9-48	11	31
B 1157	10-48	2-49	4	19
C 334	11-47	12-48	13	30
B 423	10-47	8-48	10	31
D 770	5-48	11-48	6	24
E 520	9-48	9-48	0	20
C 624	11-47	8-48	9	30
B 689	10-47	6-48	8	31
B 712	11-47	1-49	14	30
C 694	11-47	6-48	7	30
B 759	9-47	1-49	16	32
C 1070	10-48	1-49	3	20
B 771	11-47	8-48	9	30
B 820	12-47	8-48	8	29

* Calculated to date of first specimen examined May 1950.

The case of E 520 was of interest in that he may have harbored the organisms for a brief period only, and, since he denied ever having symptoms of gastrointestinal illness, it was believed that he acquired his infection through contact with another carrier. This man had reported aboard about three months prior to his first culture and that assignment was his first shipboard or sea duty; his first 5 cultures were negative, and 19 specimens taken aboard ship after the single positive were negative. These observations do not necessarily mean that he never excreted the organism again, however, because in another carrier (C 1276) a single positive specimen was obtained during the shipboard studies and only one positive was found after 26 successive negatives in the current series (table 3).

From 6 of the 24 men (25.0%) in the current preliminary series of specimens examined, *Sh. flexneri III* was recovered at least once, and these carriers were retained for further study. Intermittency of positive cultures was again evident and a summary of the alternating negative and positive phases[3] is presented in table 3. From a total of 344 specimens examined during the 70-day preliminary period, 153 (44.5%) were positive for *Sh. flexneri III*. The number of positive phases ranged from 1 to 11 and the negative periods from 2 to 11. The highest numbers of consecutive positive cultures in positive phases were 1, 3, 5, 10, 13, and 20 respectively, while the maximum numbers of negative specimens between positive cultures were 3, 4, 8, 10, and 18. In the case from which a single positive culture was recovered (C 1276), it is difficult to establish the maximum length of negative phases but, in addition to the 33 negative specimens indicated in table 3, there were 17 negative examinations during 19 days in the second series just prior to treatment; it is of interest also that, during the shipboard studies, only one positive culture was obtained from this man. The possibility that the single positive culture recovered in this instance may have been due to a technical error, such as mislabeling, was considered but was ruled out by careful investigation.

After the 6 carriers returned from leave, 17 rectal swab specimens were examined prior to antibiotic treatment;

TABLE 3.—*Alternation of positive and negative phases during serial culturing for* Sh. flexneri III *of 6 men in preliminary series.*

Study no.	Cultural phases*																								Totals				
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Positive cultures		Days	Culture phases	
																									No.†	%		Pos.	Neg.
B 438	13	1	1	2	1	2	1	1	2	3	1	2	9	3	1	1	8	1	4	1	2				43	71.7	70	11	10
	15	1	1	3	1	2	1	2	2	3	2	3	10	4	1	1	9	2	4	2	2				60				
B 1008	5	1	2	‡			3	2	5	2	1	1	1	4	1	2	4	3	3	2	1	1			26	59.1	51	10	9
	6	1	2				3	3	5	3	1	1	1	5	1	3	4	4	3	3	1	1			44				
C 1276		26	1	33																					1	1.7	70	1	2
		30	1	39																					60				
B 581		4	1	1	3	4	1	18	2	5	1	2	1	2	1	7	1	1	2	1	1	1			14	23.3	70	10	11
		5	1	1	3	5	1	21	2	6	1	3	1	2	1	9	1	1	2	2	1	1			60				
C 1308	5	1	20§	1	1	8	2	3§	1	2	12	1	3												44	73.3	70	7	6
	6	1	23	1	1	10	2	4	1	2	14	1	4												60				
D 410	1	1	1	3	7	7	1	1	2	1	10	8	1	3	1	1	1	10							25	41.7	70	9	9
	1	1	1	4	8	8	1	2	2	1	12	9	1	4	1	1	1	12							60				

* Numerators = number of cultures taken during the phase; denominators = number of days in the phase.
† Numerators = number of positive cultures; denominators = number of specimens examined.
‡ On emergency leave for 18 days.
§ Hospitalized from 18th culture day in 2nd positive phase until 1st culture day of 4th negative phase, during which anal papilla was removed.

all men denied having any illness or medication while absent from the study. From only two of the men, however, were cultures positive for *Sh. flexneri III* obtained in this short, second series; 7 out of 17 specimens taken during 19 days were positive in one case (B 438), and 16 out of 17 were positive in the other (C 1308). It is difficult to determine whether the other 4 men had spontaneously ceased to be carriers in the period of 27 days during which they were not cultured. With the exception of one individual (C 1276), the maximum length of negative phase previously observed was 18 days, but wide variations in this respect were evident.

The results of culturing these 6 carriers provide additional and relatively precise data regarding the possible minimal duration of asymptomatic *Sh. flexneri III* infection; a summary of this information is given in table 4. By reference to table 1, it can be seen that the first three men shown in table 4 were not ill at the time of probable initial infection; it is possible, therefore, that they may have become carriers at dates later than those assumed. The last three men listed in table 4, however, were ill at the times indicated, so that more confidence may be placed in fixing the probable dates of their initial infections. It is of importance to note that the duration of infection in these 6 carriers ranged from 20 to 56 months, or from over one and one-half to nearly 6 years. From two of the carriers, positive cultures were still being recovered at the time antibiotic therapy was started, so that it is quite possible their carrier condition would have persisted indefinitely without therapeutic intervention.

TABLE 4.—*Duration of* Sh. flexneri III *infection in 6 men detected as carriers in 1948 and 1950.*

Study no.	Month-year of probable initial infection	Month-year of last positive culture	Months duration of infection*
B 438	5-47	9-50	40
B 1008	10-48	7-50	21
C 1276	10-48	6-50	20
B 581	2-48	7-50	29
C 1308	10-47	9-50	35
D 410	11-45	7-50	56

* Calculated to date of last known positive culture.

Cultural studies on the two Hospital Corpsmen who presented themselves for study and treatment were not as extensive as in the experimental group. In one case, 4 cultures positive for *Sh. flexneri III* were recovered from 5 specimens examined during 6 days prior to treatment; 37 specimens examined during 56 days after beginning of therapy were all negative. In the other case, treatment was begun after a single specimen examined was found positive; 15 specimens taken during 18 days after treatment was completed were all negative.

Testing of 5 strains of *Sh. flexneri III* against sulfadiazine and certain antibiotics was done to obtain evidence that the infecting organisms were satisfactorily sensitive to the agent chosen for treatment of the carriers and to confirm previous information concerning the high degree of resistance of the carrier strains to sulfadiazine. Tests with paper sensitivity disks provided tentative evidence as anticipated; results of the standard in vitro determinations are presented in table 5. As can be observed, all 5 strains were markedly resistant to sulfadiazine, which confirmed and extended previous data.[1] All cultures, however, exhibited satisfactory in vitro sensitivity to chloramphenicol, the antibiotic selected for treatment of the carrier condition. It has been stated[4] that in vitro susceptibility of bacteria,

4. McLean, I. W., Jr., Schwab, J. L., Hillegas, A. B. and Schlingman, A. S. 1949, Susceptibility of microorganisms to chloramphenicol (chloromycetin). J. Clin. Investigation, **28:** 953–963.

including *Shigella*, to chloramphenicol concentrations of 10 μg/ml or less is an indication of possible clinical application.

During the search for antibiotic-producing intestinal bacteria, approximately 300 isolates of Gram-positive organisms were examined; none of these displayed any inhibition of *Sh. flexneri III* when grown together on a suitable medium. Cultures of coliforms possessing possible antibiotic activity against *Sh. flexneri III* are still under study. It is of interest to note that no colonies of *Sh. flexneri III* were observed on blood agar plates that were not isolated from the more selective enteric mediums.

TABLE 5.—*Sulfadiazine and antibiotic sensitivity of 5 carrier strains of* S. flexneri III.

Drug used	Cultures examined and minimal inhibitory concentrations of drugs in μg or units/ml				
	B 438-1	B 1008-1	B 581-1	C 1308-1	D 410-1
Sulfadiazine	10,000 μg	10,000 μg	10,000 μg	10,000 μg	10,000 μg
Chloramphenicol	2.5 μg	1.25 μg	1.25 μg	1.25 μg	1.25 μg
Aureomycin	1.25 μg	1.25 μg	0.62 μg	1.25 μg	1.25 μg
Polymixin B	0.62 μg	0.62 μg	0.31 μg	0.62 μg	0.62 μg
Streptomycin	50.0 units	50.0 units	50.0 units	50.0 units	50.0 units
Bacitracin	50.0 units	50.0 units	50.0 units	50.0 units	50.0 units
Penicillin	50.0 units	50.0 units	50.0 units	50.0 units	50.0 units

Clinical observations.—Results of clinical observations and laboratory tests incident thereto were all essentially negative insofar as determining a focus (or foci) of infection was concerned. The bowel mucosa was found to be normal in each case by examination through the sigmoidoscope to 20 or 24 cm. Anoscopic examinations revealed nothing of significance except a mild papillitis in one individual from whom an anal papilla had been removed earlier. It is of interest that the longest negative cultural phase observed in this case (C 1308, 8/10) occurred subsequent to removal of the anal papilla, but thereafter his cultures were more consistently positive than any of the other 5 carriers. This form of papillary hypertrophy was not observed in the other cases in the group. Swab specimens of the mucosa taken at 18 to 24 cm through the sigmoidoscope were negative for *Shigella* in 4 cases, but *Sh. flexneri III* was recovered from a 24 cm specimen from the 5th individual; material for culturing was not obtained by this method from the other carrier.

Specimens of bile obtained by biliary drainage from the 6 carriers were negative for *Sh. flexneri III* when cultured on suitable mediums. That these organisms could be recovered from the bile, if present, was demonstrated, however, by seeding minute amounts of the homologous strain in the specimens taken from one individual. Samples were removed immediately and after standing at room temperature for 4 and 22 hours and cultured on the same primary mediums routinely used; *Sh. flexneri III* was recovered from samples taken at all three intervals.

Results of the clinical examinations failed, therefore, to provide adequate evidence of a focus of infection in these 6 carriers. The failure to obtain positive cultures from biliary drainage specimens suggested that the gall bladder was not involved. Whether anal papillae represent one focus of infection was not determined.

Treatment.—No untoward side effects of chloramphenicol administration were observed, although 4 of the men reported some increase in bowel move-

ments during the course of treatment. In a recent article, Long[5] states that while toxic reactions from this drug are uncommon, frequent loose stools may occur. Return to normal in this respect was prompt after cessation of treatment in three of the cases. One individual, however, reported a persistence and increase in severity of symptoms for approximately 6 weeks; at this time, he was again referred to the hospital where laboratory examination revealed a rather heavy infection with *Hymenolepis nana*. Treatment for this condition was carried out with oleoresin of aspidium according to the procedure outlined by Bockus.[6] Subsequent stool examinations were negative and the previous symptoms disappeared.

Following completion of the chloramphenicol treatment, rectal swab specimens from each man were examined according to the procedures used in the two pretreatment series; especial care and alertness were exercised in searching the primary plating mediums for colonies of *Shigella*. In no instance during 60 successive examinations in this final series was any culture of *Shigella* found. It was apparent, therefore, that under the conditions of this study, the infections with *Sh. flexneri III* in these carriers had been terminated. As noted above, similar treatment of the two Hospital Corpsmen was apparently equally effective.

DISCUSSION

The problem of the shigellosis carrier has been recognized for many years, but additional information concerning certain aspects of the condition has been desirable. Over 30 years ago, Fletcher and Mackinnon[7] made a study of dysentery carriers and called attention to the intermittency of excretion of Flexner bacilli, the usual good health of such individuals, and the implications of such circumstances. One of their subjects persisted as a carrier for at least 18 months. Emphasis was placed on intermittency as a chief characteristic of persistent infections, evidence for which was "excretion of bacilli for a few days, sometimes in large numbers," after which there were long intervals in which the organisms could not be found; one individual had an intermission of 34 days. Hudson[8] reported a case of persistent infection which he believed had a duration of about three and one-half years. More recent observations concerning intermittency include those of Van Gelder et al[9] and Philbrook et al.[3]

The observations currently reported confirm and extend those previously made in that, even with the small group studied, wide variations in the length of negative cultural phases were found. In one individual only one positive culture was found in 60 successive specimens examined (1/60), while at the other extreme 44 positives were recovered from another man from the same total (1/1.36). For the group as a whole, 1 out of every 2.2 specimens was positive as compared with 1 of 5.46 reported by Fletcher and Mackinnon. From another aspect, it is of importance

5. Long, P. H. 1950, The clinical use of antibiotics. I. Pharmacology and toxicology. Armed Forces M. J. **1**: 928–934.
6. Bockus, H. L. 1946, Gastro-enterology, Vol. III, Philadelphia, W. B. Saunders Co.
7. Fletcher, W. and Mackinnon, D. L. 1919, A contribution to the study of chronicity in dysentery carriers. Med. Res. Comm., Special Report Ser. No. 29, HMS Stationery Office, London.
8. Hudson, N. P. 1922, A case of persistent infection with B. dysenteriae (Flexner). J.A.M.A. **79**: 1676–1677.
9. Van Gelder, D. W., Daines, W. P. and Fischer, G. L. 1947, *Shigella* carriers with special reference to their therapy, including the use of streptomycin. Am. J. Trop. Med. **27**: 225–231.

to recall that from one carrier a series of 20 successive positive cultures was observed. The longest negative phase noted was 33 specimens during 39 days in the preliminary series, although to these figures could be added 17 negative specimens during 19 days of the second series. No evidence was obtained to suggest that the intermittency in recoveries of positive cultures might have been due to reinfections rather than to a periodic shedding of the organisms during a continuous infection.

It is of significance that the maximum duration of *Sh. flexneri III* infection in the presently reported series was 56 months, or nearly 5 years, while the minimum observed time in a persistent carrier was approximately one and one-half years. It appeared possible, furthermore, that the carrier condition could last indefinitely in certain individuals unless interrupted by adequate therapeutic measures.

It would be of great value if a focus of infection in asymptomatic carriers could be determined. Manson-Bahr[10] stated that the majority of apparently healthy carriers suffer from ulceration of the intestinal mucosa and that mucus retention cysts may arise as the direct sequel of chronic bacillary dysentery. Banks, in the volume by Bockus,[6] accepted this explanation and, in addition to agreeing that such cysts represented foci of infection, advanced the idea that periodic breakdown of the cysts released organisms into the colon, thus accounting for intermittency. In the clinical studies of the 6 carriers herein described, no evidence was found of ulcerations or retention cysts. The lower 24 cm of the bowel mucosa was normal in all cases except for a mild papillitis in one instance. The removal of an anal papilla in this single case failed to eliminate the carrier state and such hypertrophied papillae were not observed in the other 5 carriers. These structures are not uncommonly found in persons free of *Shigella* infection and are usually thought to be of nonspecific origin. In only one instance was a swab specimen, taken through the sigmoidoscope at 24 cm, positive for *Sh. flexneri III*; it is possible that this result did not represent infection above this point, since organisms from the anal region may have adhered to the tip of the instrument during its introduction and passage. The failure to recover *Sh. flexneri III* from the specimens of bile examined was not unexpected, since invasion of the blood stream is rare; in only one case were there any symptoms of gall bladder involvement, and all specimens of bile taken at the time were negative for the organisms.

No direct evidence for a focus of infection was obtained, therefore, in these studies. In view of the absence of noticeable ulcerations or other lesions in the intestinal mucosa, such sites of proliferation of organisms in these 6 cases appeared unlikely. These observations and the failure to obtain positive swab specimens of the mucosa through the sigmoidoscope at 24 cm in 4 instances suggested that the reservoir of infection might be in the rectal canal, perhaps in the mucosal folds just above the sphincter. Positivity of the rectal swab specimens is consistent with such a hypothesis.

The negative results with 60 successive rectal swab specimens taken after treatment indicated that chloramphenicol in the amounts employed completely eliminated the *Sh. flexneri III* infection in the carriers. The effect was prompt in that no positive cultures were found beyond the day the first dose of anti-

10. Manson-Bahr, P. 1945, The Dysenteric Disorders, ed. 2, Baltimore, Williams & Wilkins Co.

biotic was administered in those whose cultures were positive immediately prior to beginning of the course of therapy. Although it may be that chloramphenicol is less likely to produce resistant bacterial strains than certain other drugs, as stated by Long,[11] it is believed that due caution should be exercised against underdosage and promiscuous use of the antibiotic. It would be most unfortunate if this troublesome strain of *Sh. flexneri III* were to become as resistant to chloramphenicol as it has to the sulfonamide drugs.

SUMMARY

1. From a group of 24 asymptomatic men who had harbored *Shigella flexneri III* over a year prior to the studies currently reported, 18 were found to be no longer carriers of the organism as determined by 60 successive rectal swab cultures. The duration of infection in the latter group was estimated to have varied between 0 and 36 months.

2. In the same series of preliminary cultures, 6 of the 24 men were found still to be excreting the organisms and were retained for further bacteriological and clinical studies and for therapeutic treatment to eradicate the carrier condition. It was possible to determine, with a fair degree of confidence, the duration of infection in this smaller group, and this was found to vary between 20 and 56 months.

3. Wide variations in intermittency of recovering the organisms were again observed; the longest positive phase was 20 cultures, and the maximum negative phase extended for at least 33 specimens. From the group as a whole, the recovery rate of *Sh. flexneri III* was one out of every 2.2 specimens examined.

4. No definite, direct evidence for a focus of infection was obtained. Proctosigmoidoscopic examinations revealed only normal bowel mucosa up to 24 cm in the cases observed; swab specimens of the mucosa taken through the sigmoidoscope at 24 cm were negative for the organisms in 4 cases. Specimens of bile when cultured on suitable mediums were also negative. Other clinicolaboratory examinations were essentially negative.

5. In vitro sensitivity tests with 5 strains of *Sh. flexneri III* isolated during the current studies provided justification for therapeutic use of the antibiotic selected. After completing the preliminary series of cultures and the clinical observations, each carrier received a course of chloramphenicol consisting of 250 mg every three hours around the clock until a total of 15 g had been administered. In no instance was a rectal swab specimen positive for *Sh. flexneri III* in a total of 60 successive examinations made after administration of the last dose in the course of antibiotic therapy.

11. Long, P. H. 1950, The clinical use of antibiotics. IV. Treatment of infectious processes. Armed Forces M. J. I: 1273–1280.

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